

27 March 1980

Foreign students: time for the government to do its sums

To judge by the pattern of previous years more than 90% of all applications for university places for the next UK academic year have now been made, and it looks, at first sight, as if overseas applicants have not been substantially deterred by the government's ill-considered decision to make this year's fees two to five times higher than last year's. The acid test, however, both for universities and other institutes of further education will come after the summer when they discover the rate of conversion of applicants into real students bearing the new fees. And since the omens do not look good, the universities are gloomily pondering the likely consequences.

There was, and still is, little evidence that much thought went into the possible consequences of last November's decision to raise the fees. Rather, the decision was part of the government's sweeping monetarist policies aimed at reducing public expenditure. The facts were that in 1978 87,000 overseas students attended UK institutions of further education (35,000 at universities) and that they were being subsidised by the British taxpayer to the sum of £100 million per annum.

In order to save the taxpayer that sum it was decided to charge overseas students the full cost of their studies. That meant £3,000 per annum for undergraduate science courses and £5,000 per annum for those undertaking medical, dental or veterinary studies. The fees for postgraduates were not stipulated, but many universities have decided to use the same rates.

The universities have been warned to expect their grants to be cut in the next academic year by an amount commensurate with their past intake of foreign students. It will be up to the universities to recoup their lost income from the students. The question is: what happens if much of the income does not materialize because the universities find themselves priced out of the market? — a distinct likelihood according to recent polls of extant foreign students in the UK that have been taken both by universities and student bodies.

There are certain prestige institutions whose very existence would be immediately threatened were their intake of overseas students to drop by the 40% to 90% that some of the polls suggest. The London School of Hygiene and Tropical Medicine with 75% foreign students and the Royal Postgraduate School of Medicine with 47% foreign students are particularly vulnerable. The Imperial College of Science and Technology and the University of Manchester Institute of Science and Technology would also have serious problems.

Even a considerably smaller shortfall in foreign students than the polls suggest will have serious consequences. In the first place it will inevitably exacerbate the academic job shortage, and make yet gloomier the prospect of easing the situation, given the demographic decline in home-grown students that begins in a few years' time.

Next, a shortage of foreign postgraduate students seriously threatens the quantity of research carried out in the UK, since many a busy academic runs his or her research project with one or

more hard-working foreign graduates. And they are irreplaceable because there is neither the financial support nor the moral justification — given current career prospects — to replace foreign graduate students with more from within these shores.

Another worrying prospect about the increase in fees is that it will not just deprive foreign students of an education in the UK, but that it will altogether hinder their chances of overseas studies. In particular, the many students from developing countries that were once British colonies who have taken advantage of the subsidised rates on offer in the UK may neither be able to afford the new fees nor to find the places or the cash to study elsewhere overseas. That would be not just a blow to the individuals and their countries of origin, but would also decrease UK influence in worldwide scientific and educational matters.

For the present, the government is adopting a policy of wait-and-see, possibly because it is not particularly concerned about the outcome either way. Thus, if it turns out that the new fees can be afforded by the majority of students, no harm will have been done. Whereas any substantial drop in student numbers can be used to justify a retrenchment of the funding of further education, a welcome opportunity for a government intent on cutting public expenditure. Presumably the government does anticipate a drop in overseas student numbers, because it has repeatedly emphasized that their number has almost trebled in the past decade, and that it currently exceeds by 15,000 the nominal quota set by the previous government. It has also argued that many of the current overseas students come from wealthy oil-producing countries well able to afford the new fees — an argument that ignores the plight of those who cannot and the fact that universities estimate that no more than ten per cent of their foreign students come from the wealthier nations.

The only concession so far made by the architects of the new policy is to provide the Committee of Vice Chancellors and Principals with enough money to provide bursaries covering the increase in fees to the 400 most able postgraduate students. Even that small but welcome concession is likely to be more than wiped out by the effects of last summer's 15% cut in the budget of the Overseas Development Administration which, in 1978, had supported in part or in whole more than 9,000 students from developing countries pursuing academic qualifications in the UK.

In the circumstances, what is urgently needed is some real idea of government thinking on the consequences of its policy. At present, even the very genuine concerns of such threatened centres as the Royal Postgraduate Medical School have been met with a stony wait-and-see response from the government, who could surely provide some concrete reassurance about their survival in the event of a serious loss of foreign students. In any case, faced with the contradictory evidence of the number of applications and the polls of students already here, it is surely time for the government to devise and carry out some real investigation of the rate at which foreign students will appear next academic year. □



United States

Research data: private property or public good?

Do scientists have a right to protect preliminary research findings from outside scrutiny? **David Dickson** reports on a growing controversy

LAST year, when a New York public interest group needed help in analysing the results of a national survey of infant-feeding practices, it turned to the Center for Disease Control (CDC) in Atlanta for assistance in putting the data through a computer.

CDC agreed to cooperate, and the data were duly stored. But being a public agency, its records are open to public scrutiny under the Freedom of Information Act. And CDC subsequently received a request from Abbott Laboratories and Mead-Johnson (a subsidiary of Bristol Myers), for access to the survey data. Ross Laboratories (a subsidiary of Abbott) and Mead-Johnson have been criticised over the nutritional value of their baby foods.

In January an administrative law judge upheld CDC's view that it lacks the legal power to deny the companies access to the data. And the centre is now being taken to court by the public interest group, the Interfaith Center on Corporate Responsibility (ICCR) to protect its data until it has been able to analyse the results and publish its own conclusions.

Two factors are complicating the increasingly sensitive problem of whether a research worker has any moral or legal rights over preliminary research data. The first is the growing economic importance of such data, for example where animal studies or clinical trials determine whether a new drug can be licensed. The second is the public's demand for information on substances likely to affect lives.

In the case of privately funded research, companies retain the right to withhold all test data supporting claims of efficacy or safety, on the grounds that these are trade secrets whose publication might have an economic impact on the product.

Moreover the Supreme Court, in a case brought by a group of physicians demanding access to data criticising the efficacy of an anti-diabetes drug, ruled last month that the raw data generated by a private laboratory under contract to the National Institutes of Health do not constitute an 'agency record' within the meaning of the FoI Act.

The main problem concerns the type of access permitted to federally-conducted research: in the case of clinical trials, for example, it can be argued that the disclosure of partial results before the trial is completed may jeopardise the final outcome.

Spurred by such concerns, NIH is seeking exemption for such data from the

Freedom of Information Act. Initially it had proposed that both epidemiological studies and clinical trials should be exempt. But following various objections, it has now developed a more restricted proposal, namely that it will be possible to withhold from public disclosure 'trend data' until it has been completed and verified (individual medical records are already protected by law).

To counter the argument that, without access to information on the conduct of the trial, the public has no check on whether it is being carried out responsibly, NIH is also proposing that exemptions will only be allowed if explicit measures have been taken to assure patients' safety.

Not all critics are satisfied. Dr Sidney Wolfe of the Health Research Group for example, which has consistently attacked the secrecy surrounding drug tests, argues that the protection of patients and the integrity of the tests can only be guaranteed through the fullest possible disclosure.

"Research workers can have an incredible bias, which may not be in the best interests of participants or intended participants in a particular trial" he says. "In general the more people who have access to a given body of data, the better".

Others argue differently, not only supporting the pragmatic claim that upsetting a large-scale blind trial may be very expensive, but also suggesting that a research worker has a moral right to present the first interpretation of data he or she has collected.

"This has been an underlying postulate of scientific conduct which is generally accepted" says Professor John Edsall of Harvard University, chairman of the

American Association for the Advancement of Science's Committee on Scientific Freedom and Responsibility.

Some also fear that, if a scientist's research notes are not protected from public scrutiny, he or she may be tempted to destroy them once the data has appeared in its final form.

The NIH's proposal that its clinical trials may, in given circumstances, be exempt from the full provisions of the FoI Act have been closely scrutinised by the NIH's Ethics Advisory Board. And following lengthy discussion of the revised proposal at a meeting of the board two weeks ago, it seems that the board will be prepared to support the proposal when it meets again in April.

It has been less convinced by arguments that information provided by private hospitals to CDC — for example on problems of infection in hospital wards — be kept secret for fear that publicity could generate undue public concern.

CDC had argued that, without some guarantee of anonymity, hospitals may be reluctant to come to them for assistance. But board members expressed the opinion that, although exemptions might be appropriate in some circumstances, this type of information was likely to have already reached the public domain by other routes.

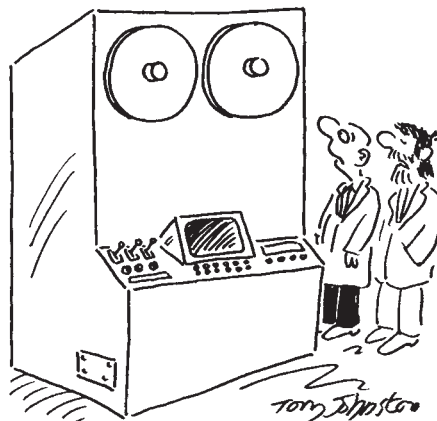
And then there is the case of the infant feeding survey results, private data whose presence in CDC's computers appears to have made it public.

Here the view that all information should be as open as possible conflicts directly with the claim that, particularly in such a sensitive area, those who have collected the data should be allowed to offer their interpretation before others try to refute it.

Indeed, in this case the two baby-food manufacturers concerned have already written to family practitioners suggesting weaknesses in the study, which was carried out to discover the extent to which lower-income families use processed baby foods, and the nutritional implications.

ICCR claims that, since it collected the data — and has agreed eventually to make it available to anyone interested — it has a right to prevent its prior use by others. And it quotes in support the practice of the World Health Organisation, namely that research data received for analysis and processing "will not be released in incomplete form to any third party without permission from the research scientist who has collected the data".

It is now up to a court of law to decide what force the traditions of the research community hold against current legal definitions of the rights of access to the results of research. □



"What makes you think the computer will be biased against processed baby foods?"

US universities are keeping pressure on federal agencies to reduce the burden of government regulation on the administration of research grants. The universities claim that over-zealous regulation has created a climate of distrust between themselves and the government.

Two reports emphasising the need for more sympathetic regulation have recently appeared in Washington, one arguing for a general relaxation of the rules governing how universities should account for the use of research funds. The other seeking institutional changes in the way that such rules are administered.

The first report, produced by the National Commission on Research, a body established in 1978 by a group of higher education institutions, says that universities should be given greater responsibility to regulate themselves. For example, rather than research workers being required to complete detailed reports of the way in which they spend their time, it suggests that there should be merely "explicit certification by individual investigators that direct salary charges to their research agreements are reasonable and fair, coupled with the federal program officer's review of the reasonableness of these expenditures for the work undertaken".

The commission, whose report appears in the 14 March issue of *Science*, also recommends that government agencies and universities should construct an option, analogous to the "standard deduction" in income tax calculations, to charge activity treated as indirect costs under sponsored agreements. The fixed percentage would be

Grants regulation: change urged

negotiated, and could either be uniform, or vary from one institution to the other.

More specific recommendations have come from the Sloan Commission on Government and Higher Education, a two-year enquiry financed by the Alfred P Sloan Foundation of New York. In general, says the Sloan Commission, support systems for university research are "fundamentally sound". But steadier funding and long-term real growth are needed: and like the NCR, the Sloan Commission criticises the excess of government regulation.

The Sloan report, which was published in Washington last week, recommends the development of a "corps of federal auditors sophisticated about scientific research and how research universities operate". It suggests that the "natural location" for a new audit agency would be in the National Science Foundation.

At present, most universities are audited by the Department of Health, Education and Welfare, according to a federal rule that auditing responsibility is assumed by the government agency which provides the largest financial support.

In recent months DHEW auditors have become increasingly critical of the way in which universities account for the use of research funds, pinpointing areas of

misuse and inadequate auditing. According to the Sloan Commission, however, DHEW's representatives "often have little experience in dealing with research institutions, receive little training, and typically stay in the assignment only briefly".

The commission suggests that the NSF is the most competent body to conduct financial oversight since it is "the only federal agency devoted entirely to research". (Although others have argued that its present close relationship to universities might preclude any credibility as an objective auditing agency.)

In other recommendations designed to strengthen federal support of university research, the commission recommends that every research grant and contract carry with it an additional 7% of the project's direct costs. These would be used in direct support of research, but on projects selected by the university and its faculty rather than the funding agency.

And in order to help alleviate the growing problem of faculty recruitment, the Sloan Commission recommends that about \$100 million a year be spent on two new kinds of fellowship to provide short-term support for PhDs. One would be a scheme of 1000 competitive national post-doctoral fellowships awarded each year, and carrying two years' support, and renewable for a further two years. The second would be a scheme of 300 national research fellowships, each carrying five years of support for research on university campuses, in federally-funded research centres and intramural federal laboratories. □

Antibiotics in animal feeds: health study 'may be impossible'

In a report that gives little direct guidance to policy-makers, a panel of the National Academy of Sciences has concluded that there is insufficient evidence to determine whether the use of sub-therapeutic doses of antibiotics in animal feeds is a significant threat to human health.

The report also states that the research necessary to establish and measure a definite risk "has not been conducted and, indeed, may not be possible."

The Academy's report seems likely to spark another round of a controversy that has simmered ever since the publication of the Swann report in the UK in 1968. This pointed to the dangers of antibiotic resistance being passed from animals to humans. Antibiotics in animal feedstuffs were subsequently banned in the UK, and later in other European countries.

Attempts to impose a similar ban in the US have been strongly opposed by the animal feedstuff and livestock industries. When the FDA announced in 1977 that it was considering such a ban, the proposal was withdrawn after a public hearing at which several companies expressed the view that there was insufficient evidence to demonstrate that such a ban was necessary

— and that if imposed it would considerably increase the price of meat.

The Academy's report, produced by a panel under the chairmanship of Dr Reuel Stallones of the University of Texas School of Public Health, provides little clue to the FDA about how it should proceed. But it does highlight areas in which more data are needed.

For example, the report says that those in close contact with animals receiving antibiotics "are more likely to harbour antimicrobial resistant *E. coli* than persons who are not exposed. However, studies do not usually indicate the type, duration and dose levels of the antimicrobials received by the animals: sub-therapeutic use was not distinguishable from therapeutic use".

Furthermore, the panel states, there are no data from which to assess the relationship between the consumption of meat from animals that received sub-therapeutic amounts of antibiotics, and the general prevalence of antimicrobial-resistant *E. coli* in human populations.

Nor, it suggests, can much help be gathered from studying the situation in Europe. "Data gathered from the United Kingdom, the Federal Republic of

Germany and the Netherlands do not indicate clearly whether restrictive regulations have actually reduced or averted the postulated hazards to human health", the report says.

And it adds that restrictions on the use of antibiotics in the United Kingdom "may well have altered the patterns of their use without significant alteration to the total amounts used or their consequences".

The committee does suggest four possible studies on individual aspects of the transmission chain — on the effects in animals, on the passage of antibiotic resistance to meat-eaters, on the effects of occupational exposures to animals, and on the human morbidity and mortality consequences of antibiotic resistance — which, it says, "would provide a useful scientific background for policy-makers".

But the panel warns that at best "the remaining gaps in our knowledge will still have to be bridged by conjecture and speculation". This is little consolation to the FDA or to Congress, which has already provided a further \$1.5 million in the Agency's current budget for a full-scale comprehensive survey.

David Dickson

Soviet Union

Incident at Military Village No. 19

LAST week the Soviet Union for the first time admitted that an outbreak of some killer infection occurred last year in Sverdlovsk. Replying to a question from the US State Department, the Soviet authorities confirmed that an outbreak of anthrax did occur, but maintained that the disease was caused by poor food handling, not by bacteriological warfare agents as rumoured in the West. Since the Soviet Union has signed the international convention banning the use of such agents, this denial comes as no surprise. What is perhaps most significant is that the Soviet authorities have admitted that some bacteriological incident did take place after almost a year of "no comment" or "nothing happened".

Sverdlovsk, on the eastern side of the Urals, is a "closed city" to which foreign visitors are not admitted. Local information reaching the West is necessarily at least second-hand, and it would be unwise to give undue credence to every detail. The details on which the US State Department based its enquiry seem to be drawn from a single Russian underground source — albeit one which claims to emanate from Sverdlovsk.

However, even one such document is worth taking seriously — if not as evidence of what occurred, at least as a pointer to local fears and responses.

According to this document, in April 1979 there was an escape of a bacterial strain called V-21 in "Military Village No. 19" on the south-western outskirts of the city. The bacteria were carried by a north wind to the village of Kashino, with only a negligible amount reaching Sverdlovsk proper.

Casualties were mainly among the military and civilian residents of "Military Village No. 19", inhabitants of Kashino, and workers at the local ceramics plant. Death typically occurred some 1-3 hours after hospitalisation, following a fever of over 42°C. Special army nurses wearing protective clothing were brought in. Bodies of those who died were not returned to the relatives for funeral ceremonies. The total death toll was unofficially estimated at over 1,000.

Official local reaction, says the report, took the form of placebo messages, which gradually escalated from "nothing is happening" at the beginning of the outbreak, to "nothing happened, and anyway, don't panic, it is all localised and under control" towards the end. Finally, the streets of Kashino village were paved with asphalt, apparently to neutralise remaining infection.

Equally circumstantial are rumours emanating from Slovakia of a similar incident involving a Soviet military base.

Here, however, there were no fatalities, only a widespread outbreak of what appears to be a new form of infective hepatitis. According to local sources, an area of central Slovakia some 70 km by 30 km is regularly used by Soviet paratroopers as a training ground. Some time in late August or early September it is alleged that one such training exercise led to the accidental discharge of bacteriological weapons. The military casualties were said to be too numerous for the existing army facilities, and the

overflow was taken to civilian hospitals in Poprad and Spišská Nova Ves.

Inevitably some of the virus was borne by the wind outside the training ground, and "several thousand" of the civilian population also succumbed. The Slovak sources stress that this does not appear to have been intended as a killer weapon — rather one to debilitate and incapacitate the local population and thus minimise resistance before the arrival of an unwished for "peace-keeping" force.

Vera Rich

Nuclear energy



Loch Doon, Scotland: UKAEA refused test drilling permission

Power dissenters through Europe

AN overwhelming majority of Swedes voted against any further extension of nuclear power in a special advisory referendum last Sunday. By a combined vote of 76% to 19% with 5% undecided, the electorate registered its approval to phase out Sweden's present nuclear programme within 25 years. Thirty nine per cent of the voters supported the Centre Party-Communist proposal to dismantle the programme within 10 years, 37% supported the Social Democrat-Liberal proposal to use present and planned capacity for 25 years as an emergency measure only, and 19% supported a Conservative option that would leave the door open for the development of uranium mining and the fast breeder.

● Swiss voters in the village of Hagedorf in the north west canton of Solothurn may have blocked effectively further nuclear development in Switzerland last week by refusing to give permission for test drilling for nuclear waste disposal. The 2,500 villagers rejected proposals by the State-backed company Nagra to drill for waste disposal sites by a 3-1 margin, despite

having voted in favour of the government's energy programme last year.

● French residents in the Brittany towns in cap Sizun claimed a victory last week when the provincial prefect at Quimper freed seven anti-nuclear demonstrators arrested during running battles with the police at protests on 29 February. A crowd of several thousand residents shouted "we have won" at the 1,500 police stationed around the Quimper courthouse. M. Jean-Marie Kerloc'h, mayor of Plogoff, called the decision "comforting". After the court decision and the pre-trial mass demonstration of 40,000 people, it is now widely believed that the central government will be forced to back down from its attempt to impose a complex of four nuclear reactors on the strongly nationalistic Breton people.

● In Finland 5,000 demonstrators, including a large number from the countryside, marched through Helsinki last week to show their solidarity with Swedish anti-nuclear protests and their opposition to the construction of a Soviet

designed 1000MW plant. The demonstration was the largest in Finland since the anti-Vietnam war demonstrations of the late 1970s. Finnish environmentalists are demanding that the contract between the state-owned Imatra Voima company and the Soviet export organisations, Atomenergoexport and all other future nuclear decisions be subjected to full parliamentary debate.

● Dutch police arrested 29 anti-nuclear demonstrators who chained themselves to the gates of the Borssele nuclear plant in Zeeland last week. The protest prevented two changes of shift at the nuclear facility. A total of 150 demonstrators, members of the Dutch anti-nuclear group "Break the Netherlands Atomic Chain" padlocked all seven gates at the plant, preventing entrance for 20 hours until their arrest and dispersal by police.

● In Britain, a second Scottish local authority has refused planning permission for test site waste disposal drilling. The Atomic Energy Authority has asked for another public inquiry to be held to try to obtain permission to drill 32 boreholes near Loch Doon, south west Scotland after its proposal was rejected by the Kyle and Carrick county council. The Conservative-controlled council feared that if granted, the proposal would lead to a detailed application for a demonstration disposal plant.

● According to figures released by the Secretary of State for the Environment in parliament recently, the UK civil and military nuclear programmes produce 100m³ of high level liquid wastes, 500m³ high level solid wastes, 450m³ plutonium contaminated waste and 250m³ miscellaneous waste each year. Some medium and low activity wastes including plutonium-contaminated material and reactor decommissioning wastes are dumped at sea.

The total waste stored at civil nuclear power stations at the end of 1979 was 20,000m³. Of this, the total accumulation of high level liquid waste was 1,000m³ at Windscale from the reprocessing of power reactor fuel and 700m³ at Dounreay from the reprocessing of fuel from experimental reactors. In addition, approximately 9,000m³ of high level solid waste and 3,500m³ of plutonium-contaminated waste are in store. The total accumulation at AEA and British Nuclear Fuel sites of medium level liquid wastes, concentrates, sludges and resins, wastes from decommissioning reactors and other plant and other medium level wastes is about 11,000m³.

The National Radiation Protection Board has spent £70,000 since 1977 to assess the radiological consequences of disposing of radioactive waste in geological formations.

Joe Schwartz

EEC

Melt-down experiment go-ahead

FRANCE has removed its objections to an experiment simulating the melt-down of a nuclear reactor, and so removed the last obstacle to the European Community's £530 million, 1979-83 programme for fusion research and support of the Joint Research Centre at Ispra, North Italy.

Italy, as part of its diplomatic activity over European research (3 January, page 3) had refused to endorse the budget of the EEC's fusion programme until France accepted the meltdown experiment, which is now to take place between 1983 and 1986 in the reactor Essor at Ispra.

France was planning its own similar experiment at Cadarache, but, says Mr Tom Doyle, head of Essor division, "Cadarache has only half the dimensions of ours" and the scale of the experiment substantially affects the results.

The US exerted pressure for the Essor experiment — called 'Super-SARA' — to be undertaken as a next step from similar, smaller tests which have been performed on the Power Burst Facility at Idaho. According to a 1979 Nuclear Regulatory Commission report Super-SARA should provide more information on melt-down than any other planned experiment.

"Essor is a heavy water reactor" Mr Doyle

told *Nature*; "the active core is 1.5 metres high, compared to a typical research reactor's 0.8 metres." Moreover there are enough channels to make simultaneous tests on 36 fuel rods (compared to 16 at Idaho).

Super-SARA will consist of 20 experiments to simulate loss of coolant in a light water reactor either rapidly, over 10-20 minutes, or more slowly (the 'small break' case as at Three Mile Island). Fuel rods will be taken to the rupture of cladding, but it is intended to leave the rest of the reactor unaffected. This is "a bit of a difficult problem" says Doyle, but not insurmountable.

The experiments are necessary, he says, because calculations are very difficult: "you have a two-phase problem under transient conditions".

The successful flurry of diplomatic activity between Italy and France over Super-SARA began last December, when the EEC Council of Energy Ministers was faced with a French veto. The French High Commissioner for Atomic Energy in the case was Jean Teillac who, as President of the Council of the European sub-nuclear physics organisation, CERN, was resisting Italian moves to 'clarify' CERN's future.

Robert Walgate

UK attacks mercury directive

THE latest draft environmental directive issued by the European Commission in Brussels concerns mercury emitted into rivers by the chlor-alkali industry — and it has come in for the now familiar sharp attack from the UK House of Lords sub-committee on the environment.

In a report published last week, the sub-committee charges that the EEC directive should not confine itself to one industry (there are other sources of mercury), should deal separately with solid and soluble mercury, and should not specify limits without indicating whether an absolute maximum or some kind of average is intended. Moreover, some of the limits are unduly stringent, says the report.

However, Britain emits more mercury into its rivers than any other EEC country, when the figures are related to chlorine production: 16.9 g per tonne of chlorine compared, say, to 3g per tonne in France.

But the basic difference between the Lords and Brussels is over the difference between "environmental quality objectives" (EQOs) as usually adopted for water in Britain, and the "emission standards" favoured by other EEC members. Although Britain has convinced the Commission to establish both for each

new pollutant considered (mercury is the second, after aldrin, dieldrin, and endrin, and cadmium will be next) the philosophies of the two are too different for them to sit easily together.

EQOs are easiest to establish where the control of river catchment areas is unified, as it is through the water authorities in the UK, and water use, defining necessary quality, a national matter. But in continental Europe rivers such as the Rhine are far from such unification.

"The apprehensions of member states subject to cross-frontier pollution" says the report "rest partly on the fact that an upstream member choosing the EQO alternative route could adopt a water use requiring less stringent standards than a neighbouring downstream member state obliged to use the water for purposes . . . which would demand higher standards".

Britain and the rest of the community are thus likely to have continuing difficulties over environmental standards; and these are accentuated by the greater scientific rigour required when setting EQOs as opposed to emission standards. "It is clear" says the House of Lords report "that the Commission has too few staff available to work on environmental proposals . . . For example, there is no expert on toxicology." The House of Lords will debate the report on 17 April 1980.

Robert Walgate

'Water pollution: mercury' House of Lords Session 1979-80 38th report of the Select Committee on the European communities. HMSO.

NEWS IN BRIEF

US-Yugoslav interferon agreement

THE National Patent Development Corporation, a private company which is planning to build a pilot plant in New Brunswick, New Jersey, to produce interferon, last week announced that it had signed an agreement with the Yugoslav Academy of Science's Institute of Immunology for regular supplies of the protein.

Under the agreement, National Patent will receive 4.5 billion international reference units of the human leukocyte within the next six months. And within that period, an accord will be reached under which National Patent will be supplied with 10 billion units a month.

Meanwhile the *Boston Globe* reported last week that research workers at the Massachusetts Institute of Technology expect to announce shortly that they have developed a method for producing interferon at only five per cent of current production costs.

The process is said to be based on research carried out in MIT's Department of Nutrition and Food Science, which has shown that human cells can be grown on small beads of starch, and that such a process can be used in particular for cells producing interferon.

Although the efficacy of the technique has yet to be demonstrated, the process has already been licensed exclusively to Flow Laboratories of Virginia. The company is currently negotiating with the National Cancer Institute to supply 50 billion units of interferon using the MIT process, which is claimed to reduce the cost of a million units from \$50 to \$2.50.

The CIA's toxic agents

BIOGEN — the European-based genetic engineering company largely owned by International Nickel and Schering Plough, which recently announced the successful synthesis of interferon — may have a less illustrious predecessor. According to information made public recently by the Church of Scientology, the Central Intelligence Agency developed a machine called Biogen in the late 1950s to manufacture toxic organisms for use in covert biological warfare.

The scientologists, who have been conducting an active campaign to discredit the activities of the CIA, say that the machine was used for 13 years, and may have produced hundreds of pounds of various biological agents and microorganisms, in particular those capable of causing undulant fever and tularemia.

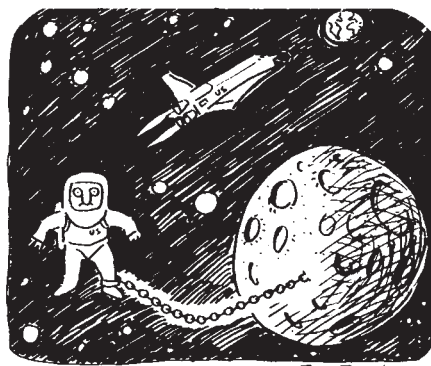
The scientologists also claim that the machine was kept in operation until 1972, three years after President Nixon had publicly renounced the use of biological weapons in warfare.

Frosch approach to space delinquency

COMMANDERS of the National Aeronautics and Space Administration's space shuttle, now due for its first flight early next year, will have the authority "to use any reasonable and necessary means, including physical force if necessary," to maintain order on board his craft, NASA administrator Dr Robert Frosch agreed last week.

Dr Frosch approved a new rule that would give the commander of the space shuttle powers to arrest any member of the space crew — including space scientists on board — and charge him or her with a crime punishable by a \$5,000 fine, a year in prison or both.

Agency officials argue that such rules were not necessary on earlier space flights involving fewer crew members and more constrained surroundings. But the space shuttle will carry seven individuals, four of whom will be civilian scientists. And according to NASA General Counsel S Neil Hosenball, the new situation demands both a formal chain of command and regulations "concerning possible criminal behaviour in space".



As a precedent, Mr Hosenball quoted the case of a technician who had been killed on an ice island in the Arctic after going berserk and threatening other members of a research team tracing the movement of ice-flows. A manslaughter charge was reversed by the Alaska Court of Appeals, which ruled that it lacked jurisdiction over a crime committed on an island that spent virtually all its time floating in the Arctic Ocean.

Oil drills escape US export control

AFTER several weeks of intense debate, the Carter administration announced last week that it is planning to tighten controls on American exports of high technology products to the Soviet Union. According to a statement issued by the US Commerce Department, the new controls will cover areas such as computers and software, manufacturing technology, and "materials critical to the manufacture of high-

technology defense goods".

The new policy follows an extensive review of the government's attitude towards high technology exports, initiated in response to the Soviet Union's intervention in Afghanistan in December. In the area of computers, for example, administration officials stated that standards will be tightened to conform to those of 1976, when restrictions of computer exports to the USSR began to be released.

One exception to the ban will be on the export of oil drilling equipment to the Soviet Union. The administration had been advised that to cut off such exports, in a field where USSR technology is said to be many years behind that of the US, would only encourage the Soviet Union to seek oil supplies in other countries, and in particular to look towards Iran.

Radiation protection on a budget

IF one man-Sievert of radiation were distributed uniformly over the British population, resultant cancers would be thinly distributed and there would be little public reaction. But if the radiation were concentrated in one town, there would be no more cancers (if response is linear to dose) but there would be an outcry because of their concentration.

Considerations such as these have led the UK National Radiation Protection Board to make estimates of the amounts that it would be cost-effective to spend to protect Britain from unit amounts of radiation dose — if it were uniformly or non-uniformly distributed. One man-Sievert uniformly spread in Britain is worth £100,000 of protection, estimates the report (though it could be a factor of five either way). But concentrated in a town of 10,000 people it would deserve protection of £40 million.

NRPB admits its figures 'have no absolute significance', but are intended to be the basis for consultation.

'The application of cost-benefit analysis to the radiological protection of the public' NRPB, Harwell, Didcot, Oxon OX11 0RQ

HSE gets Coalite report

THREE reports discussing the effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) on workers at the UK firm of Coalite and Chemical Products were handed over to the Health and Safety Executive (HSE) last week. Two weeks ago *Nature* reported Coalite's reluctance to make this information public (5 March, page 2). The report now in the HSE's possession discusses immunological, biochemical and chromosome studies of workers exposed to dioxin at Coalite between 1968-71. An HSE spokesperson is quoted as saying that the Executive is now "assessing and appraising" the reports.

FEATURES

Distorting the epidemiology of cancer: the need for a more balanced overview

The politics of cancer are becoming increasingly polarised with environmentalists taking one extreme view and industrialists the other. **Richard Peto** argues that a more dispassionate and disinterested approach is required for a realistic assessment both of the risks from environmental carcinogens and of the preventative regulations needed

THERE exist, both in the general environment and in certain occupations, chemical or physical agents which increase the likelihood of human cancer. Our political response to this would, in an ideal world where sufficient knowledge was available, depend on the direct and indirect costs of the various possible measures of control, and on how many cancers each such measure would prevent. In the real world, estimates of the direct financial costs of the control of particular agents can easily differ by one or two orders of magnitude, the indirect costs may be grossly exaggerated, or some major indirect costs may be completely overlooked. Worst of all, we have in general no remotely reliable estimates of the numbers of cancers which particular legislative controls would prevent. Consequently, there is wide scope for pressure groups to have considerable influence on public policy.

Historically, the most powerful pressure groups in US society have been the large financial interests, which have almost always put financial advantage before human health. Over the past few years the major tobacco companies have launched massive sales drives in the Third World, which if successful will kill millions of people. (About one in four regular cigarette smokers is killed prematurely by smoking.) In the US, where cigarettes cause well over 100,000 deaths every year from lung cancer or chronic obstructive lung disease, the tobacco industry refuses to accept in public that cigarettes cause either disease, let alone to collaborate in serious public information, and no epithet can suitably describe the collective efforts of their advertisers.

No other industry kills people on anything like the scale that the tobacco industry does, but where other industries have been found to cause cancer (or dust-induced lung disease) in their workers or in

the consumers of their products, their immediate response has usually been to delay acceptance of the findings, to minimise their relevance to current practice, and in general to delay or obstruct any hygienic measures which will cost money. Even when human danger has been unequivocally demonstrated, industrial consortia may actively lobby for controls so weak that (as with the new UK government regulations limiting inhaled asbestos to 1 fibre/ml from 1981) they leave no reasonable safety margin. Large amounts of money are available to mount press or TV publicity campaigns about the homely apple-pie virtues of asbestos, journals financed by the tobacco industry run populist articles which misrepresent research results to lay readers, and some US television journalists are explicitly told always to censor all reference to the dangers of smoking.

Even the scientific literature is not immune from distortion by financial interests. The decades-long argument that "threshold" dose levels of carcinogens must exist below which the general population is absolutely safe has not been entirely motivated by the scientific plausibility of the hypothesis. With increasing understanding of the derivation of tumours from single cells acted on by mutagenic carcinogens, industry is slowly abandoning "threshold" arguments in favour of arguments^{1,2} (where the biological fallacies^{3,4} are somewhat better concealed by the mathematics) that thousandfold reductions in dose can conveniently be "statistically guaranteed" to produce a million-fold or some other enormous reduction in risk.

Even if the scientists who propound such models are disinterested, industrial endorsement of them is not. Much excellent toxicology may be done by industry, and many industrial scientists and managers may be directly and honestly concerned with the prevention of hazards. But so many examples of financially-motivated bias exist that the motives and work of industrial scientists and

consultants are inevitably distrusted.

Over the past quarter century, the "environmentalist" camp in North American society has pressed for (and achieved) stricter control of particular carcinogens in the work-place, the general environment, and the diet. Usually, their efforts have been directly resisted by the relevant financial interests. Inevitably, in view of the scantiness of reliable quantitative knowledge about the cancer risks to man of nearly all carcinogens, the argument has polarised. In the politics of cancer, each side takes a very extreme position. Industry usually argues for the irrelevance to man of animal or *in vitro* cancer tests, or minimises the quantitative hazards and exaggerates the costs of control. The environmentalists usually exaggerate the likely hazards and are largely indifferent to the costs of control.

'The most powerful pressure groups in the US have been the large financial interests which have almost always put financial advantage before human health'

The vacuum of reliable scientific knowledge is such that each side can find scientists who will maintain in courts, in public hearings or in the scientific literature whatever is politically convenient, and it is important to recognise that scientists on both sides of this debate now have career interests at stake in it.

A particularly good example of the biased writings of politically active environmentalists is Samuel Epstein's *The Politics of Cancer* (Sierra Club Books, 1978, revised in Anchor Press, 1979). Epstein outlines, for university-educated but not necessarily science-educated readers, his view of our present state of knowledge about prevention of death from cancer. He concludes that the cure rates for the major cancers have not been improving much over recent years, but that we do know enough now about the causes of cancer for the testing and regulation of environmental contaminants to prevent the majority of US cancer deaths, and that the main obstacles to our doing so are political rather than scientific. He therefore reviews the scientific and political circumstances surrounding a

Richard Peto is a reader in cancer studies at the University of Oxford. This article developed from a review of Samuel Epstein's book, The Politics of Cancer

dozen or so disputed consumer products or occupational hazards, providing much fascinating political detail, and then describes at length the internal politics of the various agencies which research, regulate and litigate in Washington.

Epstein's book has been written to inflame political passions against environmental carcinogens, and parts of it are well worth reading. However, the political punch is often achieved at the expense of scientific accuracy and balance. Despite this, the book has already gained wide and apparently uncritical acceptance even among scientists. For that reason, it is worth considering some of the misrepresentations of scientific evidence which it contains, and some of the more general defects in the environmentalist perspective on cancer.

First, a few details. Epstein's book is very useful as a source of reference to original papers, but it is not in itself a reliable secondary reference because the material presented is so often distorted. Sometimes this distortion is due to the effects of Epstein's vigorous campaigning style on his scientific judgement and is, perhaps, forgivable; at other times it appears to be deliberate, which cannot so easily be forgiven.

For example, consider the (by now generally agreed) finding that incorporation of about 5% by weight of saccharin into rat diets gives a few rats bladder cancer, but has no generally accepted effects on any other type of cancer. By human standards, 5% represents a vast saccharin intake (and so, of course, the industrial "Calorie Control Council" have tried to shrug off these findings). To refute the common reaction that "anything given in large enough doses will cause cancer in animals", Epstein's chief argument is to report that 0.01% saccharin has also been shown to be carcinogenic. In support of this extraordinary claim, he presents in tabular form the control and 0.01% data for selected cancers from certain multi-group feeding experiments, leaving out the observations from those same experiments which would

have refuted it (see table). This appears to be a deliberate attempt to deceive the reader. It is not a casual slip in a 600-page book, as Epstein devotes twenty pages and two full-page tables to saccharin. His entire table 6.4, entitled "Tumours other than the bladder in rodents fed saccharin", appears to be so subject to artefacts of selection that it provides no evidence that saccharin does cause any rodent tumours other than in the rat bladder.

Saccharin is not an isolated example of bias; indeed, I found that in many places where he discussed data with which I was familiar inaccuracies were present, almost always in the direction of accentuating the need for battle with the devils of industry.

Turning to more important matters, Epstein asserts that any benefits from the "less dangerous" cigarettes which the tobacco industry has developed will be outweighed by people increasing their consumption to get more nicotine, and he is therefore in many places particularly scathing about (or even downright opposed to) research into changed cigarette composition. This is a distorted perspective on one of the more promising immediate means of preventing fatal cancers, a quarter of which in America are currently due to smoking.

Smokers of "less dangerous" cigarettes have already been found in various epidemiological studies to have disease rates which are materially lower than smokers of other cigarettes^{6,7}; at autopsy they have far fewer "pre-malignant" histological changes in their bronchi⁸ and, perhaps due to the changes in cigarette composition 10 or 20 years ago male lung cancer death rates in early middle age are now decreasing in North America, in Britain and in Finland.

After lung, the next commonest fatal cancers in the US are those of the breast and the large intestine, for which the most striking epidemiological finding to date is a 90% correlation between fat consumption in different countries and their breast or colon cancer rates (see figure; similar correlations exist for colon cancer¹⁰). Epstein suggests that dietary fat may

'Scientists on both sides of the environmentalist debate now have career interests at stake'

merely be acting as a vehicle for fat-soluble pesticides and industrial chemicals, which predictably emerge as his villains yet again. However, his suggestion is implausible: countries with high levels of contamination do not stand out from the general cancer/fat relationship, colorectal and breast cancer were common long before the widespread use of pesticides, and in experimental animals high-fat or low-fat diets can greatly enhance or reduce the risk of cancer¹¹. Something more interesting is waiting to be discovered in our diet than simple contamination by carcinogens.

The fat-associated and smoking-derived cancers collectively account for more than half of all cancer deaths, and people concerned with cancer politics should try to understand them considerably more accurately than Epstein appears to. One can list many other instances where Epstein's content or style are misleading or unbalanced. For example:

- The discussion of alcoholism and cancer is entirely erroneous because of failure to standardise properly for age.

- One of Epstein's chapters ends: "Eisenhower warned against the growing national threat of the military-industrial complex. The medical-industrial complex now appears to be as serious a threat".

- There is idealisation of the value of long-term animal tests with concomitant denigration of the value of the Ames test and other short-term tests. This is one of Epstein's most inexplicable errors of scientific judgement, unless he wants cancer tests to be difficult and expensive for industry.

- He is irritatingly puritanical, sneering at "the plastic age which symbolises how the value of our lifestyle has been degraded".

- He seeks by stylistic tricks to attribute to oral contraceptives some of the established hazards of hormone replacement therapy and of diethylstilboestrol.

- He claims that cancer costs the US economy over \$25 billion per year. (In fact, cancer must be prevented for humane, not for economic reasons; without cancer, there would be three or four million more retired North Americans to support, costing over \$25 billion per year).

And so on. He seems so certain about everything — how can anybody be justified in being so certain about so many details? Sometimes I know he's wrong, but more often (especially in the many places where inconclusive scientific results are presented as established fact) I know that nobody knows for sure. Lewis Thomas once wrote, in another context: "The sceptics in medicine have a hard time of it. It is much more difficult to be convincing about ignorance concerning disease mechanisms

Table: Data from OTA report⁵ on the tumour sites selected for presentation in Epstein's book to substantiate the claim that 0.01% saccharin is carcinogenic. Groups selectively omitted in Epstein's book are marked with an asterisk. These omissions substantially alter the implications of the original data.

Saccharin dose (% in rat diet; these doses have no material effect on longevity)	Male or female lymphosarcomas (FDA, 1948)	Female breast (FDA, 1973)	Male breast (FDA, 1973)
0 (control)	0/20 ⁺ (0%)	6/26 (23%)	6/29 (21%)
0.01	8/14 (57%)	14/30 (47%)	14/25 (56%)
0.1	5/16 (31%)*	13/34 (38%)*	9/27 (33%)*
0.5	2/15 (13%)*	—	—
1	1/18 (6%)*	12/30 (40%)*	8/27 (30%)*
5	10/17 (59%)	12/27 (44%)*	7/25 (28%)*

*Omitted in Epstein's book.

⁺In addition to these 20, since some other sweeteners were being tested concurrently, 34 other control animals were studied in the same experiment, and the 1977 OTA review of these data, considering all the control animals to be equivalent, cited 9/54 control lymphosarcomas. (0/10 saccharin controls of each sex were studied, not 0/20 as Epstein inadvertently indicated.)

than it is to make claims for full comprehension, especially when the comprehension leads, logically or not, to some sort of action. When it comes to serious illness, the public tends, understandably, to be more sceptical about the sceptics, more willing to believe the true believers."

Epstein (like many others) is unjustifiably definite about three major issues. He seems certain that even after the effects of cigarettes have been allowed for, Americans live in an era of rapidly increasing cancer rates. But trends in recorded cancer death rates (and, perhaps more so, recorded incidence rates) over the past quarter century are biased upwards (since the cure rates for the major cancers have not materially improved) by improvements in medical care and cancer counting. These improvements have

'The NCI-NIEHS report shows how a group of reasonable men can collectively generate an unreasonable report'

affected all sectors of US society, but most particularly old people and previously poor people, especially blacks. Epstein, along with most other US commentators, does not allow for these biases even to the limited extent of restricting his attention to trends in age-specific mortality rates among middle-aged whites, where they might be expected to be least prominent.

Among middle-aged whites during the past quarter century, cancer cure rates have not materially improved, but some US cancer mortality trends seem to be genuinely down (stomach, cervix) while some seem to be genuinely up (pancreas, melanoma, and certain lymphomas). I can discern no net pattern other than that due to the massive effects of smoking on lung cancer, although no extension to 1978 yet exists of the excellent 1950-67 trend analyses in NCI monograph 33. In Britain, the same is true¹² as long as we examine mortality in middle age. (Interestingly, although Epstein emphasises that bladder cancer death rates among whites in the industrial north-eastern US are high, this may not be chiefly due to any current industrial hazard, for they have been decreasing both relatively and absolutely for a quarter of a century¹³.)

Epstein also seems certain that the majority of human cancer is caused by chemical and physical agents in the environment and could be prevented by their testing and regulation. There is no sound scientific basis for this certainty. Since the cancers (lung, breast, large intestine) which are commonest in the US are rare in certain other countries, and *vice versa*, they probably really are preventable, but (see figure) not necessarily by regulation of any environmental pollutants

or food additives. Indeed, one of the most intriguing animal findings is the effect in many studies of gross aspects of diet on cancer; for example, mice randomised between 5g of food once daily and *ad lib* consumption averaging 6g/day of the same food, had respectively 8% and 64% spontaneous mammary tumours¹⁴, and the role of dietary fat¹¹ has already been discussed.

Thirdly, Epstein seems certain that at least a quarter of all cancer deaths are attributable to occupational exposure to carcinogens. There is as yet no good evidence for hazards of anything like this magnitude, and there is clear evidence that many of his particular claims are exaggerated. For example, he says that asbestos has led to approximately 50,000 deaths per year in the US from cancer and related disease (referencing a paper by Selikoff¹⁵ which actually neither makes nor implies any such claim). This is sheer nonsense; of 50,000 asbestos deaths, several thousand should be certified as being due to pleural mesothelioma, yet only several hundred per year are thus certified.

Likewise, Epstein cites with approval (indeed, he describes it as being of "epochal significance") Health, Education and Welfare Secretary Califano's absurd 1978 estimate, in a speech to labour union leaders, that as many as half of the 8-11 million workers who have been exposed to asbestos could develop serious diseases such as cancer or asbestosis. In his second edition, Epstein draws extensively on Califano's source, a curious but extremely influential document which has been circulating privately for the past year or more. This unpublished typescript, with no listed authors, was prepared by a working group of nine well-known scientists including Arthur Upton and David Rall, the National Cancer Institute and National Institute of Environmental Health Sciences directors,

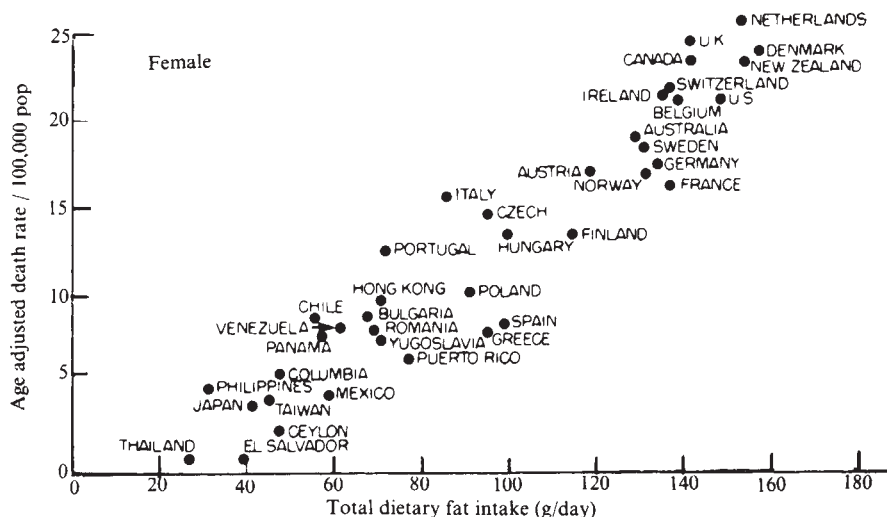
'Present day cancer rates and trends are probably not dominated by occupational carcinogens or environmental pollutants'

both of whom still seem more ready to defend than to repudiate it.

Entitled "Estimates of the fraction of cancer in the US related to occupational factors", it shows how a group of reasonable men can collectively generate an unreasonable report. The group multiplied the 12 million workers exposed in any way in 1972-4 to any of asbestos, As, Ni, Cr, benzene or petrochemicals by the very large risk factors (e.g. fivefold lung cancer excesses) typical of much more extensively exposed industrial populations, finally multiplying again by "4-5" to allow for the additional cancers that will be caused by these six agents among workers exposed to them at times other than 1972-4. (In casual disregard of dosimetry, this whole process is very like predicting 100,000 occupational lung cancer deaths per year among non-smokers due to their exposure to other people's cigarette smoke at work, "since 20% of heavy smokers get lung cancer").

These assumptions "predict" over 200,000 extra respiratory tract cancers per year in the near future due to the above six agents alone (and are the sole source of the currently widespread prediction of "20-38%" of cancers being due to occupation). But, in reality, fewer than 90,000 males/year get respiratory tract cancer in the US (and most of these are due to cigarettes and not to occupation at all), and moreover the US male rates in the first 25 years of working life are currently decreasing! A prediction of 100-200,000 extras due to the above six agents is clearly

Figure: data⁹ plotting for 39 countries age-versus total dietary fat (animal or standardised breast cancer death rate vegetable).



very unrealistic.

The unreality of the whole method is further highlighted by the fact that the assumptions "predict" 7,300 × 4.5 nickel-oxide induced occupational lung cancers/year. Since nickel refining causes a lot of nasal as well as lung cancer, we would presumably then also soon "expect" something like 13,000 nasal cancer death certificates per year among nickel workers. Nickel has already been widely used for decades, so many thousands of these should presumably already be evident. In reality, each year in the US population there are only 300-odd male and 200-odd female nasal cancer death certificates. The majority of these are not ex-nickel workers and no marked trends are evident. This demonstrates the unsoundness of the NCI-NIEHS methodology.

Unfortunately, such exaggerated estimates are so much what many people want to believe of modern society that they have now achieved a life of their own, and although they are utterly without foundation they are quoted widely, repeatedly and reputably both in the lay and scientific press. In Geneva the International Labour Organisation reportedly endorsed them, as did the Toxic Substances Strategy Committee in their recent draft report to the US president¹⁶, and they have been used in OSHA to emphasise the need for stricter laws. Most recently, the UK trade union the Association of Scientific, Technical and Managerial Staffs (ASTMS) released a policy document which appears to have been strongly influenced by Epstein's book, and which is notable for its prominent but erroneous assertion, derived solely from the foregoing unpublished US typescript, that 20-40% of current UK cancer deaths are caused by occupational carcinogens.

Epstein now claims that these exaggerated estimates seriously underestimate the impact of industrial carcinogens. There is obviously a valid political need to exaggerate the importance of the preventive measures we can already

death rates¹² should be more widely known.

My criticisms of Epstein's science, however, must be viewed in the light of the continued resistance of many industries to reasonable controls. One has only to read some of his descriptions of industrial behaviour to see where his passion comes from, and a suitably sceptical reader could derive much important information from the dozen or so detailed case-histories of particular carcinogens that make up the bulk of this book. But, although I cannot prove it, I suspect that environmentalists as a whole (including Epstein and those US and British trade unions which have already modelled their public statements closely on his book) would be better

'The politics of cancer is dominated on both sides by exaggeration'

respected and could therefore press more effectively for controls in particular instances if their overview of cancer was more balanced in the following ways:

- if they accepted that present-day cancer rates and trends are probably not dominated by occupational carcinogens or environmental pollutants (especially since they could still warn that future rates might perhaps be so dominated unless present-day exposures are regulated²⁰);
- if their chief concern was with the identification and control of the few major determinants of human cancer (or, in an industrial context, the few major industrial carcinogens) rather than the large multitude of sins which Epstein denounces;
- if they discussed the costs and benefits of research into the control of toxic substances in the context of other possible ways of improving longevity (bad diet and tobacco in rich countries, malnutrition and infective and parasitic diseases elsewhere);
- if they took the costs of imposing restrictions on society more seriously. Epstein's general assertion is that the total direct and indirect costs of failing to regulate usually exceed the real costs of the testing and regulation, but I suspect that this is a slogan to avoid political embarrassment rather than a carefully researched conclusion;
- if they accepted that for most toxic chemicals we have both qualitative and vast quantitative uncertainty about the health benefits of restriction.

Particularly, even if the sort of grossly biased interpretation which Epstein applied to the animal saccharin data is avoided, the results of animal experiments simply are not an uncomplicated key to human hazard identification. First, moderate alteration of gross aspects of the diet of animals greatly modifies their spontaneous tumour onset rates¹¹ (so, for example, increasing the sugar content of rat diets may increase their cancer rates

more than the equivalent amount of dietary saccharin would have done¹¹). Also, any chemical which causes proliferation or necrosis in any organ that is subject to spontaneous cancers is likely to modify the onset rate of tumours in that organ, and since the aim of most animal experiments is to study a dose which is nearly, but not quite, sufficient to cause significant weight loss or mortality within three months, it is not surprising that so many chemicals at such doses can cause cancer in animals.

However, it may be that where adversary politics operate one needs views at both extremes in order to get a balanced outcome. And there is a possibility that over-emphasis on the avoidance of scientific error would emasculate the environmentalist passions and merely lead to my own rather inactive conservatism. (After all, no scruples about scientific certainty of safety usually precede the widespread introduction of new chemicals, so should the imposition of prudent restrictions require absolute proof?)

Environmentalists with biased judgments and a quasireligious certainty of right will fight more battles than any reasonable sceptic would do, and even if their victories confer 10 or 100 times less benefit on humanity than they imagine, they will in the long run probably do more good than harm — unless they materially reduce food production, distort research priorities, or direct attention away from the smoking problem in the process. Epstein justly quotes, from the 1st century AD, the Plutarch chronicles: "He who in time of faction takes neither side shall be disenfranchised". So, I feel, should both environmentalists and industrialists who suppress or deliberately misinterpret data, but they probably won't be. For the moment, the 'politics of cancer' is dominated on both sides by exaggeration. □

'For most toxic chemicals we now have both qualitative and quantitative uncertainty about the health benefits of restriction'

implement, but in the long run we do also still need to discover the main preventable causes of cancer other than smoking. Bearing both these needs in mind, there are limits beyond which it is not even politically wise to distort the science of cancer, and some of today's exaggerations may well transgress those limits. Reliable reviews of the known and suspected causes^{17,18,19} of human cancer and of trends in British

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CORRESPONDENCE

Liberties with energy

SIR,—Gerald Leach (*Nature*, 21 February, page 714), suggests that the pursuit of a high conservation/low energy strategy would not touch personal liberties. No doubt this can be so, but if this policy is maintained it is difficult to believe that a really major effect on fuel conservation is likely to be achieved. There have been many cases in industry in which fuel conservation has actually been of financial advantage to the firms concerned, but it seems likely that the proportion of firms for which this is true will decrease as time goes on, and that many firms will find the value marginal if not negative.

In a depressed and highly competitive period it is very unlikely that firms will at all readily undertake extra expenditure which is not immediately productive, and firms exerting freedom of choice are likely to stay as they are. The likelihood therefore that we shall do too little too late on a voluntary basis is really quite large, in which case Gerald Leach himself says that energy conservation may have to be forced through by controls on individuals.

I am not myself too much bothered by this. The interference with civil liberties would be little more than the controls exerted by fire, smoke control and town-planning regulations. At the same time, I can see no reason to suppose that any protection of nuclear installations that may be necessary should have a worse effect on civil liberties than the similarly necessary protection of many industrial activities such as chlorine production or the manufacture of nylon precursors at Flixborough. Freedom to walk into other people's factories does not come high on my list of vital freedoms, whether they are processing nuclear wastes or military weapons or indeed cigarettes. A severe shortage of power with strict rationing would seem to me a more serious limitation of my liberties than would the fact that I could not get into a nuclear processing works without passing somebody carrying a revolver.

If there is a genuine risk of a terrorist attempt to kill many thousands of people in a single major incident, we should have to introduce a great many controls much more onerous than the guarding of a few well defined nuclear establishments in limited areas. One would, for example, need to control an appreciable area of South Essex and North Kent to ensure that a few saboteurs with a rocket launcher should not blow up the Canvey Island stores of liquified natural gas some dark night with a westerly breeze.

Yours faithfully,

J. H. FREMLIN

Department of Physics,
University of Birmingham

CEGB on radiation

SIR,—Further to the item headed 'UK Radiation Study Overestimates Safety' (14 February, page 614). I wish to point out that the radiation dose data supplied by the Central Electricity Generating Board to the Health and Safety Executive and also published elsewhere does include the doses received by workers other than those directly employed by the CEGB. We consider it important to include the radiation doses of all classified workers in the dose data relevant to the operation of CEGB nuclear power stations. Only by using such data can sensible comparisons be made between the doses received at nuclear power stations in the UK and those in other countries.

You may be interested to know that the total dose received by non-CEGB classified persons at all CEGB nuclear power stations in

1978 was 236 man-rems, and that received by CEGB personnel was 1399 man-rems. The mean dose per man for each category was 168 mrem and 257 mrem respectively.

Yours faithfully,

R. B. PEPPER

Health and Safety Department, CEGB,
London, UK

Lost in translation?

SIR,—Your readers may know that Nobel Prize lectures are translated into many languages. Mine, delivered in 1977, described the growth of our understanding of non-crystalline semiconductors and contains the following sentence. "The discovery of this property of glasses" (namely that they cannot be doped) "certainly makes Kolomiets" (Leningrad) "one of the fathers of the branch of science that I am describing, as were others in Eastern European countries, notably Grigorovici in Bucarest and Tauc in Prague." Grigorovici remains in Bucarest, I believe retired; Tauc is in America.

For reasons unknown to me, the translated version published by the Czechoslovak Journal of Physics, with the permission of the Nobel Foundation, omits the last two of these names. A letter to the editor-in-chief from the Nobel Foundation, dated 7 November 1979 and asking for an explanation has not yet been answered.

Yours faithfully,

NEVILL MOTT

Department of Physics, University of
Cambridge, UK

Insignificant figures

SIR,—Uncritical recording of estimations with numbers that include insignificant figures is a plague in scientific literature. Although technological advancements make possible increasingly more accurate measurements, overall precision is limited by the less accurate step involved. Moreover, modern calculators tend to increase the number of figures without any relation to significance.

To quantify this abuse we have examined a selection of first class journals looking for clearly insignificant figures. The results exceeded our expectations: in the issue(s) corresponding to one month of 1979 all journals examined had several papers with insignificant figures. The journals are: *Eur. J. Biochem.*, *J. Biol. Chem.*, *Nature*, *Proc. Natl. Acad. Sci. USA*, and *Science*. For illustration, five examples taken from the above sampling are listed below (followed by appropriately rounded numbers in brackets):
enzyme activity: 913,856 U (914×10^3 U)
enzyme activation: $1005 \pm 80\%$ of control (10 fold activation ± 1)
glycogen in muscle: $22.43 \pm 2.89 \mu\text{mol/g}$ ($22.4 \pm 2.9 \mu\text{mol/g}$)
one type of cell in mice spleen: $104,662 \pm 18,937$ ($105 \times 10^3 \pm 19 \times 10^3$)
weight of fed female ticks: $297.26 \pm 63.6 \text{ mg}$ ($297 \pm 64 \text{ mg}$)

From extrapolation of this small sample it seems that over 10% of currently published scientific papers contain insignificant figures.

To obtain useful information from these multifigured numbers the reader has to do some mental operations (even if largely unconsciously), to eliminate from consideration the irrelevant figures, keeping three at the most. Would it not be much better to weed out the inordinately large numbers once and for all from (computer) calculation to publication? Insignificance usually runs parallel to irrelevance. Results should be both analytically significant and scientifically relevant; everything else would be noise. For instance, the liver of a young rat may weigh in

a balance as 6.2345 g; but because of the uncertainties on how much is really "the liver" (vascular pedicle, blood, and other factors), only 6.23 g would be relevant in most cases. Four or more figures would generally be mere pseudoprecision, even if analytically significant.

The rampant pseudoprecision of most results given with 4 to 6 figures must be stopped. It is suggested that authors should carefully consider the significance of the results so that only the last given figure is tentative. This practice will usually limit, in the presentation of results, the number of figures to 3, and in many cases to 2 or even 1. Obviously this norm does not apply in the case of serial numbers, dates, etc. For arithmetic means, with non-negative data, if the coefficient of variation ($\sigma/\mu \times 100$) is greater than 5, the mean should be expressed with the same number of significant figures as the individual data, since such a dispersion does not justify, in general, a greater precision. Only if it is smaller than 5, an additional significant figure could be appropriate. Exponential forms (i.e. 914×10^3) will aid and simplify in most cases the final presentation of results with a number of figures limited to the significant ones.

Finally we propose to the editors of scientific journals to include in the instructions to authors a statement that manuscripts with obviously insignificant figures would not be accepted, perhaps adding that manuscripts with results with more than 3 figures should have an explicit (and acceptable) justification in the text or in the accompanying letter to the editor. Scientific objectivity would benefit, and the work of the scientists would be easier. Implicitly, this policy would recognise the fact that some scientists spend months or even years improving conditions to add one significant figure to a constant. Moreover, scientific publishing would be a little less expensive, nowadays a not insignificant consideration.

Yours faithfully,

ALBERTO SOLS
JOSÉ LUIS CARRASCO

Autonomous University of Madrid, Spain

Exhibitionism

SIR,—Your correspondent Jim Ritter, in an otherwise perceptive feature (3 January, page 6) on the proposed French science museum at La Villette, displays his ignorance of what contemporary museums have been doing during the last two decades. Far from being sources of magical effects — 'black boxes with pushbuttons' — they have developed a vast array of exhibits and programmes that make science more understandable and accessible to public visitors. Modern science museums demystify science and technology and suggest that, although complex, science can be understood, controlled, and even enjoyed.

The Association of Science-Technology Centers, to which more than 100 museums belong, would be glad to provide an itinerary of museum travels on several continents to bring him up to date.

I would recommend a tour through the tactile gallery and the visual perception exhibits of the Exploratorium (San Francisco), the human reproduction and sexuality exhibit at the Ontario Science Centre (Toronto), the plumbing exhibit and the *Food for Life* exhibit at the Museum of Science and Industry (Chicago), or the exhibition *It's About time* at the Franklin Institute (Philadelphia).

Your faithfully,

MICHAEL TEMPLETON
Association of Science-Technology
Centers, Washington DC, US

NEWS AND VIEWS

A closer look at Saturn's magnetosphere

from S. W. H. Cowley

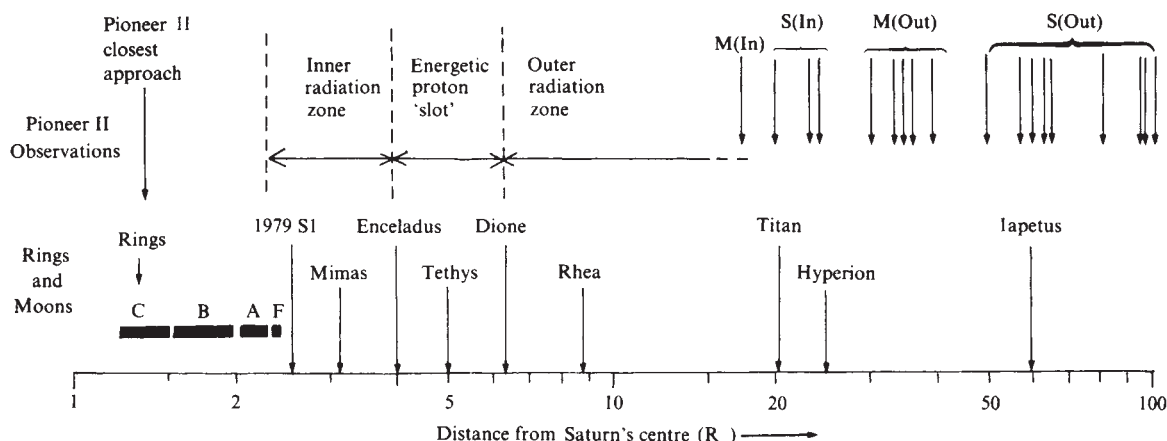
At the beginning of September, 1979 US spacecraft Pioneer 11 undertook the first close survey of the planet Saturn, approaching within 21,000 km of the visible cloud-tops. Pioneer 11 was launched in April, 1973 and had previously made the second Jupiter fly-by in December, 1974, one year after Pioneer 10. All eleven of the spacecraft's instruments which had been operating at Jupiter were still functioning 5 years later at Saturn, and sent back data on the planetary magnetic field and charged particle environment, as well as observations of the planet and its rings and moons in the ultraviolet, visible and infrared bands. A recent issue of *Science* (207; 1980) was devoted to the Pioneer results and here I shall concentrate on the discoveries made by the particle and magnetic field detectors on Pioneer (see Wolfe *et al.*, Smith *et al.*, Simpson *et al.*, Van Allen *et al.*, Trainor *et al.*, Filius *et al.*, Gehrels *et al.* and Acuna & Ness in that issue of *Science*). These included the fourth magnetosphere in the Solar System (after

Earth, Jupiter and Mercury), a new Saturnian ring and at least one new moon.

Saturn had been suspected for some time to possess a strong dipolar magnetic field, such that the planet would be surrounded by a large cavity in the solar wind plasma (which pervades the Solar System) containing the planetary field — a magnetosphere. In 1975 radio bursts from the planet, characteristic of magnetospheric plasma processes, had been detected and an equatorial field strength of ~ 1 gauss inferred (compared with ~ 0.3 gauss at Earth and ~ 4.1 gauss at Jupiter), a value which agreed well with deductions from an empirical 'magnetic Bode's law' which relates the magnetic dipole moment to the planet's spin angular momentum. Using ~ 1 gauss for the surface field strength it was estimated that the magnetosphere would extend to $\sim 40 R_S$ (R_S = Saturn's radius = 60,000 km) on the dayside, the boundary (magnetopause) location being determined by the point where the solar wind dynamic pressure and

the planetary magnetic pressure just balance. The magnetosphere would then always incorporate both the rings of Saturn and all the moons out to Hyperion, as indicated in the accompanying figure.

Pioneer 11 approached Saturn very nearly along the noon meridian (along the Saturn-Sun line), but did not in fact enter the magnetosphere until it was $\sim 17 R_S$ from the planet, as indicated by M(In) in the figure, less than half the expected distance. Subsequent magnetic measurements soon revealed the primary cause to be a rather weaker planetary field than anticipated. The equatorial surface field is ~ 0.2 gauss, with the same polarity as Jupiter's field, but opposite to that of the Earth and Mercury. In swinging round Saturn, nearly in the equatorial plane, Pioneer 11's trajectory was deflected through nearly 90° , and the spacecraft moved out from the planet nearly along the dawn meridian. In this direction the magnetopause is expected to be at a rather larger distance than at noon, and due to



Logarithmic scale plot of the position of Saturn's rings and moons, and their relationship to Pioneer 11 observations. The new F ring and moon (1979 S1) have been included, but further small moons may exist between the orbits of Mimas and the outer edge of the A ring. The D ring inside the C ring, reported from Earth observations, apparently does not exist, and no definite detection of a tenuous E ring extending out to 5 or 6 R_S was made. The moon Phoebe at $216 R_S$ has been omitted. Symbol M refers to the magnetopause (the outer boundary of the magnetosphere) observed during the noon inbound (In) and dawn outbound (Out) passes of Pioneer 11, while S indicates detection of an upstream bow shock in the solar wind.

boundary fluctuations five crossings were observed between 30 and 40 R_S . In all, Pioneer 11 spent some 2½ days within Saturn's magnetosphere.

A most surprising aspect of the magnetic data is that Saturn's field shows no detectable periodicity at the planetary spin period (~10 h), implying that the dipole moment vector is aligned within ~1° of the spin axis. This observation sets an important new constraint on dynamo theories of the origin of planetary fields, as it had generally been supposed on the basis of the other planets that a tilt angle of ~10° might be fundamental. It also frustrates attempts to measure accurately the internal rotation period of the planet.

Properties of the magnetosphere

In general, the structure and properties of magnetospheric plasmas depend very strongly on the nature of the plasma flow within the magnetosphere. Simple estimates before the Pioneer encounter showed that the overwhelmingly dominant flow in the case of Saturn (and Jupiter) should be simple corotation with the planet. This is unlike the Earth's magnetosphere, where a solar-wind driven convection rather like that in a falling raindrop is usually dominant, and where only a rather small 'core' of dipolar field lines near the Earth corotate with the planet. The difference arises mainly from the much higher dipole moments of Jupiter and Saturn compared with Earth, combined with their faster rotation rates.

In a corotating magnetosphere the only direct source of plasma is cold ions and electrons from the planet's ionosphere, together possibly with a lunar contribution if any emitting moons happen to lie within it (for example the volcanic moon Io in Jupiter's magnetosphere). This thermal plasma will be the dominant component in terms of number density inside such magnetospheres. However, more energetic charged particles originating mainly in the solar wind can become trapped in the outer part of the magnetic field if the flow fluctuates, as is always the case in practice, and the fluctuations will then diffuse these particles deep into the corotating region. An equilibrium energetic particle radiation belt will then be formed inside the magnetosphere (like the Van Allen belt inside the Earth's corotating 'core'), in which diffusion steadily feeds particles inwards from the outer source into the internal sinks (for example, absorption by the planetary atmosphere, or by moons and ring material).

The Pioneer spacecraft were not adequately instrumented to measure the dominant thermal plasma component with particle energies in the eV to keV range, but some information can be extracted from

the solar wind instrument under favourable circumstances. At Saturn, corotating thermal plasma was nearly continuously observed by Pioneer 11 during the inbound pass and distinct flux peaks were observed at the distances corresponding to the orbits of Dione and Tethys. The ionic component of this plasma appears to be mainly O^+ (or possibly OH^+) such that the source may be sputtered or photodissociated ices originating at these bodies.

Much more information is available on the energetic radiation belt particles, measurements by four instrument packages being made in the energy range from several tens of keV up to several tens of MeV. The flux of protons at fixed energy initially increases steeply with decreasing distance from the planet (as expected from inward diffusion, since particles gain energy proportional to the field strength as they move inwards). However, a peak is reached at ~7 R_S and then fluxes fall precipitately by more than a factor of 100, reaching a minimum value at the orbit of Enceladus at ~4 R_S . Absorption by Dione, Tethys and Enceladus (see figure) may be significant in forming this proton 'slot', but particle precipitation into Saturn's atmosphere due to scattering by waves excited in the thermal plasma may have a major role.

Inside the orbit of Enceladus energetic proton fluxes again rapidly rise with decreasing distance, reach a secondary peak at ~2.7 R_S before finally falling by more than three orders of magnitude to below detectability at the outer edge of the A ring. Within this inner zone of proton fluxes deep and narrow absorption features occur at the orbit of Mimas at 3.09 R_S and also at 2.52 R_S , corresponding to a previously unknown moon, designated 1979 S1, which had been detected a few hours previously on Pioneer 11 images. In addition, absorption features associated with the F ring (also newly seen on these images) were observed between 2.34 and 2.37 R_S , together with a flux plateau at 2.81 R_S which may correspond to absorption by a rather smaller new moon. The proton flux increases seen on the inside of these absorption bands seem too sharp to be consistent with the simple inward diffusion picture, and it is likely that a second source of energetic protons contributes to the formation of the inner zone, possibly the debris of cosmic ray interactions with Saturn's atmosphere and ring material. The energetic electrons show many features which are similar to the protons, but only the lower energy particles are lost in the 'slot' region. Fluxes above ~1 MeV continue to increase across the slot, if at a reduced rate, before falling to very low levels at and within the inner edge of the A ring. In general, the particle fluxes measured while the spacecraft passed under the rings were by far the lowest observed since the instruments had left the laboratory.

Pioneer's near miss

A final extraordinary event took place during the ~4 minutes in which Pioneer 11 crossed the 1979 S1 moon absorption band inbound to Saturn. For ~10 s a deep depression in the fluxes was observed by all instruments capable of such resolution which were then above background, implying that Pioneer 11 had actually passed through the flux tube containing the moon, avoiding collision by only a few thousand kilometres. The width of the flux 'hole' indicates a lunar radius of ~100 km. Similar sharp features were seen in the F-ring absorption zone, due either to more moons, or to non-uniform distribution of ring material. A final possible lunar-related disturbance was seen on the outbound pass at ~20 R_S when Pioneer 11 was ~6 R_S downstream from Titan (Saturn's largest moon) in the corotating flow. Large amplitude oscillations were observed in the magnetic field for about 2 h, possibly indicating a hydromagnetic wake produced by Titan. The corotation speed at these distances is ~200 km s⁻¹.

In conclusion, the Pioneer 11 mission to Saturn has provided a wealth of new information about the particle and field environment of the planet but leaves many questions only partly resolved, particularly as regards the thermal plasma and its sources, the nature of Titan's interaction with the magnetosphere and the existence of small moons inside the orbit of Mimas. We will not have to wait too long for some of the answers. Voyager 1 will make the second Saturn fly-by in November 1980, while Voyager 2 will reach the planet in August 1981, en route to Uranus. Meanwhile Pioneer 11 will continue on its way to become the second spacecraft to escape the Solar System, roughly in a direction opposite to that of Pioneer 10. It will be tracked and data on the interplanetary medium acquired into the middle 1980s. □



100 years ago

The work of casting the lenses of the great refracting telescope of the Paris Observatory has already begun at Feil's establishment. The founding of the flint disc has taken five days, and the annealing a full month. A like operation will soon take place for the Bishofsheim Observatory instrument.

A correspondent of *La Nature* sends that paper a photograph of a curious phenomenon met with in the cold of December last. It shows a bottle which contained a solution of nitrate of silver (1 per cent). The cork is forced out and imprisoned at the extremity of a long cylinder of ice, due to increase of the volume of the mass in freezing.

From *Nature* 21, 25 March, 500; 1880.

Monoclonal antibodies and immunity to malaria

from F.E.G. Cox

UNTIL the middle of the 1960s there had been very little success in immunising laboratory animals against malaria parasites and many workers felt that this meant that the possibility of a vaccine against human malaria was remote or even non-existent. The past decade, however, has produced abundant evidence that immunisation against these parasites in laboratory animals is not only possible but is in fact remarkably easy. This is unfortunately not really due to any major scientific breakthrough but to the judicious use of suitable combinations of strains of parasites and hosts in which the balance of the immune response is tipped in favour of the host. It is probably as difficult today to immunise random-bred strains of laboratory mice against *Plasmodium berghei* as it was 10 years ago, but avirulent parasites, such as *P. yoelli* and *P. chabaudi*, in certain strains of inbred mice are relatively easy to protect against even when the parasites have been secondarily selected for virulence.

Those working with primate malarias are even less fortunate for they have no avirulent parasites to choose from and are also limited to a small range of random-bred hosts. For the reasons outlined above it is possible to argue that many rodent malaria models are unrealistic but, nevertheless, the fact that protective immunity against any kind of malaria can be induced artificially does open the possibility that the mechanism involved might be relevant to the human disease.

In this context it is important to distinguish between the experimental host and the parasite itself. Undoubtedly man, monkeys and strains of mice differ widely in their ability to handle a complex antigen such as a malaria parasite. On the other hand, all malaria parasites are intrinsically similar in that in the life-cycle the sporozoites, injected into the blood by a mosquito, undergo a single multiplicative phase in the liver resulting in the release of merozoites that enter red blood cells to undergo a second phase of multiplication (or schizogony) resulting in further merozoites that invade fresh red cells to repeat the cycle until the host dies or mounts an effective immune response. The sexual stages of the life-cycle are largely irrelevant or peripheral to protective immunity although immunisation against gametocytes inhibits development in the mosquito (Mendis & Targett *Nature* 277, 389; 1979). In practice, many workers short-circuit the life-cycle by passaging infected blood thus eliminating the sporozoite and liver stages. Biochemical and ultrastructural studies suggest that

there are no fundamental differences between the malaria parasites of man and laboratory animals and, in view of the similarities in the life-cycles, it seems reasonable to assume that, as far as the parasite is concerned, what happens to it in an immunised mouse or monkey is likely to be similar to what happens to it in man.

The three major (and only possible) candidates for a specific vaccine are the sporozoite, the blood schizont and the merozoite. These three forms possess stage-specific antigens so the possibility of one of these inducing any immunity against the others is remote. The schizont has been widely used in experimental work but, notwithstanding the light that such studies have shed on our understanding of immunity to malaria, its practical potential is limited. This is mainly because there is considerable evidence to suggest that malaria parasites undergo some kind of antigenic variation and that any immunity produced is not only stage but also variant specific (Brown *Ciba Foundation Symposium* No. 25, 35; 1974).

This leaves the sporozoite and the merozoite, neither of which seems to be affected by the problems of antigenic variation (Cohen *Proc. R. Soc. Lond. B.* 203, 323; 1979). Sporozoites are logical targets because they are present before any clinically apparent infection. The use of irradiated sporozoites as antigens has been pioneered by Professor Ruth Nussenzweig at the New York University Medical Center and promising results have been obtained in mice, monkeys and man. The arguments against the use of sporozoites are that they probably need to develop in the liver to induce immunity and, as the protection is stage-specific, a single parasite entering and maturing in the liver after challenge will initiate an infection. Furthermore, sporozoites are at present difficult to obtain and multiple immunising doses have to be given. The alternative approach, a merozoite vaccine, has been developed by Professor Sydney Cohen and his colleagues at Guy's Hospital, London. This has also been successful in primates but the arguments against this approach are that there is no protection against the stages in the liver and that the vaccine is not effective unless administered in fairly large quantities with an adjuvant. The supply of merozoites for the preparation of antigen has been facilitated by the development of effective *in vitro* cultivation techniques (Trager & Jensen *Nature* 273, 621; 1978).

Even using the most favourable combinations of host and parasite, it is clear that all that can be done in terms of immunising animals with sporozoites or merozoites has been done and that the next stage will be to identify, isolate and purify the actual antigens involved in protection.

Biochemical techniques for the isolation of such antigens are time consuming but there is already evidence that potentially important antigens occur on the surfaces of sporozoites (Nardin & Nussenzweig *Nature* 274, 55; 1978; Aikawa *et al. J. Protozool.* 26, 273; 1979) and merozoites (Deans, Dennis & Cohen *Parasitology* 77, 333; 1978).

An obvious approach to the identification and characterisation of antigens likely to be of importance in protection is to use monoclonal antibodies produced by hybrid cells one of which has been derived from a lymphocyte actually producing antibody in an infected or immunised animal. Monoclonal antibodies to complex antigens are notoriously difficult to prepare but recently two groups of workers have reported considerable success. Yoshida, Nussenzweig, Potocnjak, Nussenzweig & Aikawa (*Science* 207, 71; 1980), working in New York, have isolated monoclonal antibodies directed against a single protein on the sporozoites of *P. berghei* in mice. This protein, which has a molecular weight of 44,000, is found on no other stage of *P. berghei* nor on the sporozoites of *P. knowlesi* or *P. cynomolgi*. Further work in progress by these workers indicates that mice bearing hybridomas are resistant to challenge with *P. berghei* sporozoites. At the Wellcome Laboratories in England, Freeman, Trejdosiewicz & Cross (this issue of *Nature*, page 366) have fused myeloma cells with spleen cells from mice immune to *P. yoelii* and produced monoclonal antibodies against the parasites in 38 out of 143 cultures. Five of these lines were expanded, one against all the stages in the blood, two against merozoites and two against infected red cells. Sera from mice carrying hybridoma lines were then injected into mice with a rising virulent *P. yoelii* infection and those against the merozoites protected the recipients from death. These sera, however, did not prevent the development of a considerable parasitaemia and were not nearly as protective as hyperimmune sera from *P. yoelii* immune mice.

Both of these sets of experiments can be criticised on the grounds that the models chosen could have been closer to the human ones and the authors would no doubt be the first to agree that these are very preliminary results indeed. They do, however, make a quantum jump in our approach to malaria immunology for they demonstrate that it is possible to differentiate between antigens that are actually targets in the immune response and those that are irrelevant. There is nothing special about the mouse malaria models and these findings can easily be applied to primate and human malarias. The way is now open for the use of monoclonal antibodies to bind particular antigens on malaria parasites that can then be freed from the antibody and used in immunization studies. It is here that the

mouse malaria model will be of less use because it depends on the particular nature of the immune response of that host rather than on the nature of the parasite.

These experiments have not shown that sporozoite vaccination is any better than merozoite vaccination nor that antibodies act exclusively, or even mainly, on merozoites. Furthermore, the failure of monoclonal antibodies directed against malaria antigens on the red cells to protect mice means that the idea that such antigens may somehow be involved in protection will have to be reinvestigated. The way ahead is clear, though, and it should soon be possible to implicate particular characterized antigens in the protective immune response and also clarify our uncertain understanding of antigenic variation. We might now have an extremely useful tool but we are still a long, long way from a realistic and acceptable vaccine. □

Death and the neurone

from Julian Lewis

THE great problems of neural development express themselves in many small mysteries. Among these is the phenomenon of motoneurone death. Vertebrate embryos generate at first an excess of motoneurons, and 50% or more of the initial set normally die, round about the time when neuromuscular connections begin to form. Why are these surplus cells produced, and what causes their death?

The phenomena are well-documented for several systems, chiefly in birds and amphibians, including the spinal motoneurons that innervate the limbs, the trochlear motoneurons that innervate the superior oblique muscle of the eye, and the parasympathetic motoneurons of the ciliary ganglion, which innervate the intrinsic eye muscles (for a recent review see Jacobson *Developmental Neurobiology*, 2nd ed., Plenum, 1978). The extent of motoneurone death can be modified in various ways. For example, the embryonic rudiment of a limb, either of a bird or of an amphibian, can be cut off at an early stage, before it has become innervated. This does not upset the generation of normal numbers of motoneurons in the spinal cord, nor the initial outgrowth of their axons; but it prevents their survival. Almost all the cells which should have innervated the absent limb die, and they die at about the time when motoneurone death would normally occur. The interpretation seems obvious: motoneurons survive only in proportion to the quantity of target tissue; muscles will accept only a limited density of innervation, and death is the fate

of motoneurons which fail in a competition to form synapses (Hamburger *J. comp. Neurol.* **160**, 535; 1975). Individuals of a species vary in their bodily proportions. The normal surplus of motoneurons would guarantee that there would be enough to innervate even limbs that were disproportionately large; cell death would dispose of any excess. Thus the final number of motoneurons would be matched to the amount of muscle.

This tempting suggestion is supported to some extent by another experiment. When an additional limb is grafted onto an embryo, fewer of the motoneurons die. The number saved from death is, however, less than the extra limb might be expected to maintain (Hollyday & Hamburger *J. comp. Neurol.* **170**, 311; 1976; Lamb *J. Embryol. exp. Morphol.* **49**, 13; 1979). Studies of the chick ciliary ganglion give other grounds for caution. There too, about half the motoneurons die during normal development, and almost all die if the early eye rudiment has been removed. But the cells that die suffer different sorts of death in the two circumstances. The normal death involves, for example, a swelling of the cisternae of the endoplasmic reticulum without detachment of ribosomes from it, whereas the death by peripheral deprivation involves detachment of ribosome (Pilar & Landmesser *J. Cell Biol.* **68**, 339; 1976).

In this issue of *Nature* (page 347), Lamb gives strong direct evidence against the idea that normal motoneurone death is due to competition for a target that will accept only a limited density of innervation. Lamb amputates one hindlimb bud of a *Xenopus* tadpole, and manages to divert the nerves that should have innervated it into the remaining hindlimb, which is thus supplied from both sides of the spinal cord. The proportion of motoneurons that die in this case turns out to be practically the same as normal, so that the total number of survivors innervating the single remaining limb is twice as great as normal. There is no sign of competition between the motoneurons for a limited territory; the limb has parking space to spare.

Another popular interpretation of motoneurone death is that it serves to eliminate errors in the pattern of connections. This idea also has its problems. In the normal course of development, even the motoneurons that will die send axons out into the periphery and, in chicks at least, they appear to send them to the right places in the periphery. This can be shown by tracing projections with horseradish peroxidase. Particular muscles turn out to be innervated by pools of motoneurons lying in distinctive and reproducible positions in the spinal cord; in the chick, this pattern is accurately established before the period of motoneurone death, and changes hardly at all thereafter (Landmesser *J. Physiol. Lond.* **284**, 391; 1978) although in *Xenopus* the projections shift somewhat at

early stages (Lamb *Devl Biol.* **54**, 82; 1976).

The most intriguing clue to the puzzle of motoneurone death comes from experiments with toxins that block neuromuscular transmission (Pittman & Oppenheim *J. comp. Neurol.* **187**, 425; 1979; Laing & Prestige *J. Physiol. Lond.* **282**, 33; 1978; Creazzo & Sohal *Expl Neurol.* **66**, 135; 1979). One might be inclined to guess that such toxins should cause, if anything, increased death of motoneurons, mimicking the effect of absence of muscle. Precisely the opposite is observed: curare, α -bungarotoxin, α -cobratoxin and botulinum toxin, given in the period when motoneurons normally die, can all prevent the death. In this they are effective so long as movement is paralysed; when the concentration of toxin falls to the point where movements can begin, the surplus motoneurons belatedly die. The normally-occurring cell death evidently depends on neural activity. The phenomenon must therefore be closely linked with another little mystery of neural development, concerning spontaneous movement: why is it that vertebrate embryos start to writhe and squirm and wave their limbs about haphazardly as soon as neuromuscular connections have begun to form (Hamburger in *Behavioural Embryology*, vol. 1 (ed. Gottlieb) Academic Press, 1973)?

The activity of a motoneurone depends on the inputs which it receives in the spinal cord; and it is after all the set of conditions under which a neurone fires, not the location of its soma, that matters for the functioning of the system. If, abandoning the notion that motoneurons die in a competition for a limited quantity of target tissue, we espouse instead the theory that they die because they have made mistaken connections, we must suppose that the mistakes lie not in the relation between the position of the soma and the choice of target muscle, but rather in the relation between the choice of target muscle and the set of central inputs to the motoneurone. A simple speculation along these lines might be that some motoneurons die because they are, in their time of firing, out of step with their fellows in a pool of motoneurons innervating a single muscle; they might feel the effects at their point of contact with that muscle, during periods of muscle stimulation. From a very early stage, there are indeed correlations in time of firing between pools of motoneurons innervating different muscles, and these become sharper as development proceeds (Bekoff, Stein & Hamburger *Proc. natn. Acad. Sci. U.S.A.* **72**, 1245; 1975; Bekoff *Brain Res.* **106**, 271; 1976). But again, the speculation encounters difficulties. It would suggest, for example, that where there is less scope for mistakes, there should be less death: that in a nucleus such as that of the trochlear nerve, which innervates only one muscle, there should be no confusion of connections, and all the motoneurons should survive. But they

don't. In the trochlear nucleus of a bird, just as in the ventral horn of its spinal cord, half the motoneurons die (Cowan in *Development and Aging of the Nervous System* (ed. Rockstein), Academic Press, 1973).

The evidence so far has not yielded a solution to the puzzle of motoneurone death; but it has at least proved that the solution will be interesting. □

Birds see ultraviolet light

from J.K. Bowmaker

HUMAN colour vision extends from about 400 nm in the violet to about 750 nm in the red. Why are we restricted to this limited range? About 80% of the electromagnetic radiation reaching the Earth's surface lies between 300 nm in the ultraviolet and 1,100 nm in the infrared, but nevertheless only part of this spectrum is available for vision since radiation with wavelengths longer than about 800 nm has too little energy to cause photochemical reactions. Thus there is available for vision a spectral range from about 300 nm to 800 nm. However, the limits of an animal's vision are determined by the visual pigments contained within the photopic receptors of the retina, the cones, and by pre-receptor filters. In man these are principally the lens that cuts off light below about 400 nm, and the macula pigment in the yellow spot of the retina. Filtering violet and near ultraviolet light reduces chromatic aberration which becomes increasingly severe at short wavelengths.

The question remains whether other species are similarly restricted. It is natural perhaps to compare the visual sense of other diurnal species with our own, on the assumption that we represent the culmination of the evolution of colour vision. But is this reasonable? The early mammals, around at the time of the dinosaurs, were probably nocturnal, resulting in their colour vision becoming largely degenerate, with many modern mammals having only limited dichromatic vision. The Old World primates, including man, are somewhat of an exception in that they have evolved secondarily trichromatic vision along with the adoption of diurnal habits. Even so our trichromacy has a 'blue' channel that is about 100 times less sensitive than the 'red' and 'green' channels. The true culmination of the evolution of colour vision in vertebrates would be expected to be found in highly evolved diurnal animals, perhaps best represented by diurnal birds and it is

within these species that we should look for colour vision making use of more of the available spectrum.

The most striking feature of the retina of diurnal birds is the presence of brightly coloured oil droplets contained within the cones that act as highly selective long-pass cut-off filters (see Bowmaker *Vision Res.* 17; 1129; 1979). The lenses of most diurnal birds are optically clear and it seems that one of the functions of the oil droplets is to limit chromatic aberration selectively at the receptor level. However, amongst the coloured oil droplets there are colourless ones that presumably transmit near ultraviolet light with the possibility that diurnal birds can detect these short wavelengths as a 'colour', different in hue from the rest of their visible spectrum.

It is known that pigeons can see in this spectral region (Wright *J. exp. anal. Behav.* 17, 325; 1972) and in a recent paper Timothy Goldsmith (*Science* 207, 786; 1980) has presented evidence that several species of humming bird can discriminate the near ultraviolet. Working with a natural population of the birds he set them a simple task of selecting artificial feeders containing a sugar solution on the basis of a coloured viewing screen set in the feeders. The birds were able to distinguish the near ultraviolet around 370 nm from darkness or from the small amount of far red light that leaked through the ultraviolet transmitting glass filter, which human observers were unable to do. The birds could also distinguish white light lacking wavelengths below 400 nm from white light from a quartz-halogen bulb that does contain near ultraviolet radiation.

The near ultraviolet sensitivity of the humming birds presumably involves cones containing an oil droplet that transmits short wavelengths but also a visual pigment that is sensitive to these wavelengths. Evidence from pigeons and chickens (Graf & Norren *Vision Res.* 14; 1203; 1974) indicates that in addition to red, green and blue-sensitive visual pigments there is a visual pigment with maximum sensitivity at about 400–415 nm. This is very close to the maximum sensitivity of the visual pigment of human 'blue' cones (415–420 nm) recently recorded by Bowmaker and Dartnall (*J. Physiol. Lond.* 298, 501; 1980), but the effective maximum sensitivity of the human cones is displaced to about 440 nm with a marked cut off below about 400 nm due to the filtering of the lens.

The other major group of animals known to have vision in the ultraviolet is the insects. Here chromatic aberration is not a problem since arthropod eyes are designed on different optical principles. These insects, principally bees and moths, have mouthparts designed to suck nectar from flowers, and many of the flowers rely on their insect visitors for pollination. Many such flowers reveal striking patterns of 'nectar guides' if photographed using ultraviolet sensitive film that are not visible

to a human observer and it is assumed that the evolution of such guides has paralleled the evolution of ultraviolet vision in these pollinating insects. It would be interesting to know whether the flowers usually frequented by humming birds have similar ultraviolet nectar guides. However, as Goldsmith says "the interplay of several spectral classes of oil droplet with several cone pigments and the presence of receptors functioning in the near UV suggests that avian colour vision possesses a richness beyond our ken". □

BL Lacertae Objects —the 'missing link'?

from a Correspondent

A RECENT study of a sample of extragalactic radio sources belonging to the so-called BL Lacertae class has shed some new light on one of the most interesting questions in extragalactic radio astronomy, namely the relationship between the radio sources associated with galaxies and those associated with quasars.

Until 1968 the optical objects found to be associated with extragalactic radio sources could be divided into two basic classes, galaxies and quasars. The galaxies have a variety of forms, basically appearing diffuse on photographs; their optical spectra are also varied, ranging from the thermal continua and weak absorption lines typical of old stars to the non-thermal continua and strong emission lines which are indicative of violent activity in the galactic nucleus. This latter type of spectrum is also typical of the quasars which appear starlike on photographs and are generally at higher redshifts than the galaxies. In 1968 an apparently new class of identification was found when the radio source VRO 42.22.01 was identified with the so-called 'variable star' BL Lacertae. The radio source itself was very unusual, showing striking, rapid variations in radio flux density and in polarization. In this respect it bore similarities to the very compact sources associated with some quasars though its variations were more extreme. However, the optical spectrum of the 'star' was apparently completely featureless showing neither emission nor absorption lines, the continuum being clearly non-thermal in origin; the optical radiation was also strongly polarized and very variable in intensity. The absence of lines in the spectrum made this very peculiar object particularly tantalizing as there was no means by which to determine its redshift and its distance.

Subsequently many more sources of this type, now called BL Lac objects, have been found and to date roughly 60 are known.

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The defining properties of the objects in this class are that their optical spectra have steep non-thermal continua with no strong emission features, there is rapid variability at optical, radio and infrared wavelengths, and there is strong and rapidly varying polarization. The objects vary quite widely in optical appearance, ranging from those which are completely stellar to those which are the nuclei of galaxies. Though it was originally thought that the optical spectra were completely featureless, careful spectroscopic work on some of these objects, particularly BL Lac itself, has revealed weak emission and absorption features similar to those found in elliptical galaxies, so that it has been possible to determine the distances and physical properties in some cases. It is now possible therefore to compare their physical properties with those of quasars and radio galaxies and to investigate their relationship to these objects.

Such an investigation has been carried out by Weiler & Johnston (*Mon. Not. R. astr. Soc.* 190, 269; 1980) who have made a detailed radio study of a large sample of BL Lac objects, examining their radio structures on scales ranging from a few thousandths of an arc second to arc minutes. Their results indicate that the majority of the BL Lac objects contain very compact cores with at least half of the total flux density and with properties very similar to the compact variable quasars. However they also find that over half of them have extended radio emission as well, with sizes and luminosities comparable with those of the extended sources associated with radio galaxies and quasars; the BL Lac objects are thus apparently indistinguishable from the radio galaxies and quasars on the basis of their radio structures. Furthermore, as the luminosities of their compact radio cores span the whole range from the relatively weak cores seen in radio galaxies to the stronger ones in quasars, they seem to form a 'link' between the quasars and radio galaxies. Further evidence of such a link comes from the fact that the BL Lac objects appear to occupy an area intermediate between that covered by quasars and that occupied by radio galaxies on a radio flux density-optical magnitude diagram.

It thus seems possible that the BL Lac objects represent a transition phase between quasars and radio galaxies. Weiler and Johnston speculate that a radio quasar may represent the early stages in the evolution of an elliptical galaxy which contains a significant amount of gas in its central regions; this gas, as well as producing the observed emission lines, is used in some way to power the central 'engine' which is responsible for the violent radio and optical activity. If this gas is used up or expelled the emission lines will disappear and the operation of the engine may become more variable, so that the quasar may evolve into a BL Lac object.

Finally as all the gas is used up, the activity may decrease still further leaving the relatively weak core sources seen in most radio galaxies.

Although this evolutionary sequence is not the only interpretation of the results it is certainly an interesting possibility. There is much scope for further work to clarify the situation. It is important to determine the space densities of the BL Lac objects to test that these are consistent on the basis of the evolutionary sequence with the already known space densities of quasars and radio galaxies. However, this analysis may be difficult if only because unambiguous selection of a sample of BL Lac objects is almost impossible as a result of the degeneration in the definition of their properties and the continuity of these properties with those of radio galaxies and quasars. More insight is likely to be gained from detailed information on the radio structures of the BL Lac objects on all angular scales; in particular comparison of the structures of the BL Lac cores with those of radio galaxies and quasars on milliarcsecond scales will be particularly revealing as it is these core sources that provide perhaps the strongest evidence for the evolutionary sequence. □

Towards the ideal seismic experiment

from Bob Whitmarsh

THE most powerful remote sensing technique used in the search for hydrocarbons is seismic reflection. Carefully controlled sources radiate acoustic energy which is reflected from sub-surface interfaces and sensed by a large number of transducers usually arranged in a linear array relatively close to, and aligned with, the source. Subsequently complex and highly sophisticated signal processing techniques are applied to the recorded signals to increase the depth range or penetration of the final reflection profile as well as its vertical and horizontal resolution. The ultimate objective of the reflection technique is to translate the observed vertical reflection times from different strata into depths and this requires a knowledge of seismic velocities in the overlying layers. During a normal reflection survey the accuracy of the velocity determinations is limited, particularly for deeper reflectors, by the length of the receiving array. This length determines the range of incident angles at the reflector for the group of rays from a single shot.

More accurate information about the deeper velocity structure can be obtained if

the distance between source and receiver is steadily changed and this type of experiment, radically different from normal reflection profiling, can be used to study reflections from vertical incidence out to the ranges where critical reflection occurs and beyond. At sea, until recently, such variable angle reflection experiments have mostly been carried out using one or more free-drifting surface sonobuoys or fixed ocean-bottom seismographs. The optimum procedure for reflection studies however is to use a source and receiver which move with equal but opposite velocities away from a fixed point (the common depth point (CDP)). Then all the reflections picked up by a towed array from a given interface originate at the same fixed area on the interface irrespective of the incident angle thereby avoiding the difficulties due to irregular interfaces. At sea the only practical solution is to use two ships, it being a great advantage if one of these is fitted with a multichannel hydrophone array. This is the procedure recently reported by Stoffa & Buhl (*J. geophys. Res.* 84, 7645; 1979) which they have used to carry out a series of deep crustal studies of oceanic areas.

The first description of a two-ship multichannel seismic experiment to study oceanic crust seems to have been that of Limond *et al.* (*Earth planet. Sci. Lett.* 15, 361; 1972). They fired a 34-km long expanding spread profile (ESP) in the Bay of Biscay. Shots fired every 800 m were received by a 2.4 km long 24-channel array thereby providing three-fold coverage. As the object was to obtain Moho reflections on the multichannel reflection profile (these were identified by working back from Moho refracted arrivals through sub-critical reflections to vertical incidence) the signal processing of the seismic traces did not proceed beyond the presentation of a record section.

The experiments of Stoffa and Buhl however were specifically designed to study the deep oceanic crust with the greatest practical precision. In such experiments both horizontal range and shot-to-receiver travel time need to be determined to within a few tens of metres and a few milliseconds respectively. Ideally, precise absolute navigation is required so that the two ships lie symmetrically about the common depth point at all times. In practice, even though the relative navigation of the ships is correct, as can be established using a ship-to-ship radio ranging system, surface currents (which may not be detectable during the experiment) can introduce important errors. Such errors probably exceed those due to dipping reflectors. Their combined effect tends to invalidate the basic assumption underlying CDP but fortunately does not necessarily negate the usefulness of ESP experiments. Except in nearshore areas covered by high quality radio-navigation systems there is at present no simple practical solution to this navi-

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gational problem. The timing precision is easier to obtain, particularly with electrically fired airguns or explosions.

In recent years advances in marine seismic work have come more from new methods of quantitative analysis and numerical modelling than of experimental technique. An example of such an analytical method is that used by Stoffa and Buhl to extract phase velocity information from the record sections. This is done on a shot by shot basis using a semblance technique to determine the phase velocity having the greatest coherence across each set of 24 traces beginning in turn at each successive digitised sample along the nearest or first trace. The technique is useful in that it enables over-lapping or 'crossing-over' signals of different phase velocity to be separated as well as giving an objective assessment of the onset times of second arrivals which might otherwise have been picked too late. From analysis of the semblance plots a precise travel time *versus* distance plot is produced for all arrivals whose semblance coefficient exceeds a given value, and this plot in turn is inverted using a derivation of a standard method to give a highly detailed velocity/depth profile of the oceanic crust.

A disadvantage of the ESP method outlined above is that the structure under each shot and receiver position may differ, whereas for a fixed ocean-bottom seismograph at least half this possible lateral variation is avoided. However some indication of lateral velocity inhomogeneity can be obtained, again using two ships, by keeping the source and receiver at a fixed distance (constant offset profile or COP) appropriate to the interface of interest. By combining COPs having different offset distances with CDP and normal multichannel reflection profiles it is possible to approach the ideal deep crustal seismic experiment. However to model the Earth fully taking account of all the constraints provided by these types of observations is probably taking the problem beyond the scope of the best computer facilities.

The technique described by Stoffa and Buhl opens up exciting possibilities for detailed deep crustal studies in the oceans. In practice however it is unlikely that there will be many researchers in a position to follow their example. Regrettably, multichannel digital recording systems, digital seismic reflection processing and two-ship experiments are expensive. But such experiments might continue perhaps on an inter-institution basis in areas of mutual scientific interest. Continental margins in particular, where the results could be of more than academic interest and where two-ship experiments are more likely to obtain the necessary funding would provide attractive targets. Such an approach has already led to a joint French-United Kingdom study of the margin southwest of the English Channel last year.

Recent advances in polarized electron sources

In his article "Polarized electrons are going around in circles" (*News & Views* **283**, 248; 1980) C.B. Lucas notes that recent advances in sources of polarized electrons should provide insights into fields as diverse as neutral currents, surface physics, and molecular optical activity. He calls attention to recent developments in atomic physics-based polarized electron sources and to the fact that beams of electrons and positrons become polarized as they circulate in a storage ring. We would like to describe some very recent advances that have been made in the development and application of solid state sources of polarized electrons.

Extraction of electrons from a ferromagnetic solid offers a conceptually elegant source of polarized electrons. The electrons are aligned by an external magnetic field and exist in densities unobtainable in aligned atom beams. A very useful source has been demonstrated¹ using field emission from a tungsten tip that has been coated with europium sulphide. At the operating temperature of 10 K, the EuS is ferromagnetic and acts as a spin filter. A polarization of 0.85 is obtained at currents up to 10 nA. Most notably, the source has very high electron optical brightness and will be useful in applications requiring highly collimated, highly focused beams.

The most recently developed² solid state source of polarized electrons uses photoemission from gallium arsenide. When GaAs is irradiated with circularly polarized light, the electrons excited from the maximum of the spin-orbit-split valence band may be polarized up to a theoretical value of 50%. GaAs is one of those special materials in which, by application of caesium and oxygen to the surface, the vacuum level can be lowered below the bulk conduction band minimum to produce the so-called 'negative electron affinity' emitter. Negative electron affinity GaAs is not only an extremely efficient photoemitter but the electrons can be polarized! When the light is modulated from right to left circular polarization, the direction of the polarization of the electron beam is also modulated, facilitating the use of very sensitive signal detection techniques.

A source of polarized electrons utilizing GaAs has been built which produces a continuous beam with a polarization of $\pm 43\%$, and a beam

current of 20 μA has been obtained for one milliwatt of incident circularly polarized light². A pulsed GaAs source has produced microsecond pulses of several hundred milliamperes at a 120 Hz repetition rate³.

In a sense, the GaAs source is the solid state analogue of the source utilizing the 'Fano effect' in caesium discussed by Lucas. The higher density of the solid provides much more intense electron beams; typically three orders of magnitude more intense than existing Fano effect sources. The energy spread of the GaAs source is 130 meV FWHM, and the effective source area is determined by the focus of the circularly polarized light, typically 0.5 mm diameter. The electron optical brightness is second only to the EuS/W field emission source.

The Fano effect in Cs can produce electron beams nearly 100% polarized. In most experiments the lower polarization of the GaAs source is easily compensated by increasing the intensity of the beam, but there are experiments, for example, where target damage is a problem, where higher polarization is required. There is hope of increasing the polarization of the GaAs source by lifting the degeneracy at the valence band maximum by (1) stressing the crystal (2) preparing epitaxially grown multilayer structures to confine the electron in one-dimensional potential wells, or by using (3) crystal field splitting in chalcopyrite semiconductors such as CdSiAs_2 or CdGeP_2 , which are ternary analogues to GaAs.

The GaAs source has already been very effectively applied at SLAC to measure the parity non-conservation in inelastic scattering of 20 GeV polarized electrons from hydrogen and deuterium³. It has been used to observe the surface magnetization of ferromagnetic nickel through the exchange interaction in low energy polarized electron scattering experiments⁴. It has also been used in polarized electron diffraction studies of single crystal tungsten⁵. The recent demonstrations of the characteristics of this type of source have led to the construction of such devices by nearly every group engaged in polarized electron scattering.

We agree completely with Lucas that there are now many exciting research opportunities because of recent advances in polarized electron sources. We believe that the new solid state source technology will play a significant part.

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ARTICLES

The narrow rings of Jupiter, Saturn and Uranus

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Recent occultation studies and spacecraft observations have revealed the presence of narrow ring systems around the planets Jupiter, Saturn and Uranus. Thus three of the four major planets are now known to contain systems of narrow rings. This article attempts to account for the origin and location of these rings.

OUR knowledge of the uranian rings is extensive and it is possible to list a large number of features which any theory of narrow rings ought to be able to account for (see ref. 1). We know little about the jovian ring system at present, apart from its location and the fact that the outer ring is only 6,000 km wide². This ring contains a bright inner ring of width 800 ± 100 km (ref. 2). Voyager 2 images have revealed the presence of a small, dark satellite of diameter between 20 and 40 km with an albedo ~ 0.05 (refs 3, 4). The orbit of this satellite lies in the ring plane and appears to define the outer edge of the rings as seen by reflected light (ref. 4 and G. E. Danielson, personal communication).

Pioneer 11 detected at least one comparatively narrow saturnian ring and possibly several small satellites, some of which may orbit within the rings^{5,6}. The radial width of the F-ring discovered by the Pioneer 11 imaging team⁶ is not greater than 800 km (ref. 6). The 3,500-km gap between this ring and the broad A-ring (the Pioneer gap) contains less material than Cassini's division⁶. The Pioneer 11 Geiger tube team⁵ detected a possible ring of width 3,600 km between 2.550 and $2.490 R_s$ ($R_s = 60,000$ km is the equatorial radius of Saturn) which may contain two satellites of diameter ≥ 170 km at 2.534 and $2.522 R_s$. They also detected a ring (F) of width 2,100 km between 2.371 and $2.336 R_s$, which may contain two satellites at 2.343 and $2.350 R_s$, and a possible new satellite at $2.82 R_s$. The fact that the two determinations of the width of the F-ring are quite different suggests that the ring is wide ($\sim 2,100$ km) but, like the jovian ring, contains a bright inner ring of width ~ 800 km.

It is well known that the 2:1 Mimas resonance lies close to the outer edge of the B-ring⁷. We point out here that the 3:2 Mimas resonance ($2.361 R_s$) lies close to the outer edge of the F-ring ($2.371 R_s$) and that the 4:3 Mimas resonance ($2.553 R_s$) lies close to the orbits of the newly discovered satellite or satellites at 2.534 and $2.522 R_s$. These calculations assume that the coefficient of the principal gravitational harmonic $J_2 = 0.01667$ (ref. 9) but neglect the mass of the rings since this is not known.

Dermott *et al.*¹ consider that the extremely narrow widths and sharply defined edges of the uranian rings imply that some kind of critical phenomenon is responsible. They suppose that each ring contains a small satellite which maintains solid particles in stable, horseshoe orbits about its lagrangian equilibrium points (see Fig. 1). They show that this model explains most of the observed features of the uranian rings. By comparison Goldreich and Tremaine⁸ suggest that each ring consists of particles confined between the orbits of two small satellites.

They show that this theory can explain how the particles can remain in precisely aligned elliptical orbits but they do not explain other features of the rings, such as sharp edges and the narrow range of widths of the rings.

The uranian and jovian ring systems, both of which are comparatively narrow, have mean orbital radii of 1.8 planetary radii. We consider it significant that both systems also lie in the Roche 'zone' defined by Dermott *et al.*¹ (see Figs 2 and 3). The location of the narrow jovian ring suggests an origin similar to that of the uranian rings. Whether this ring consists of a number, possibly a large number, of discrete rings or whether it is continuous is not yet known. We suggest that the jovian ring is the product of the disintegration of a satellite that entered the Roche zone, probably as a result of orbital evolution due to tidal dissipation, and that large numbers of small particles are now maintained in horseshoe orbits about the lagrangian equilibrium points of the remanent chunks. The recent observation of a small satellite in the outer edge of the rings supports this view. Collisional, as well as tidal processes were probably involved in

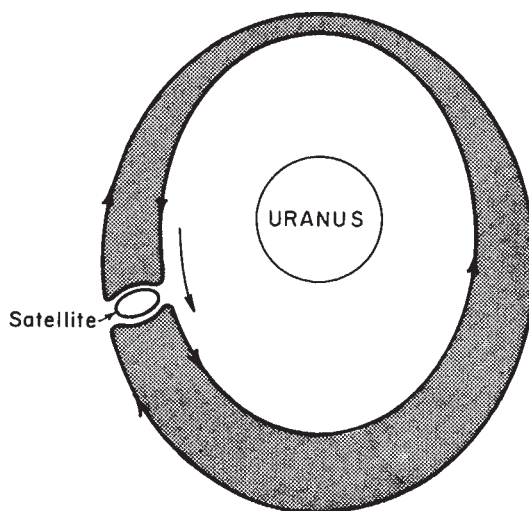


Fig. 1 Dermott *et al.*¹ suggest that each uranian ring contains a small satellite which maintains particles in stable, horseshoe orbits about its lagrangian equilibrium points. In their model, loose solid particles leave the satellite surface and enter orbits closely similar to that of the satellite (which can be both eccentric and inclined to the equatorial plane of the planet). It is the gravitational force of the satellite in a resonance with the ring particles which then provides the critical phenomenon needed to define a narrow, sharp-edged, asymmetric ring.

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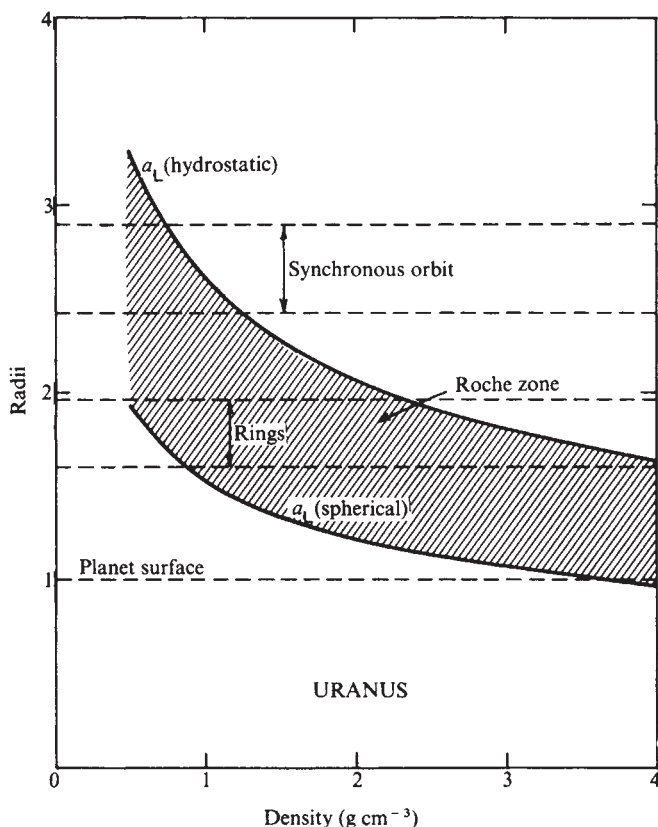


Fig. 2 Roche limit a_L for Uranus as a function of satellite density. a_L (hydrostatic) applies to a satellite that can relax to hydrostatic equilibrium and a_L (spherical) applies to a spherical satellite. Dermott *et al.*¹ refer to the zone between these two extremes as the Roche zone. It is in this zone that a satellite is expected to lose solid particles from its surface. The location of the synchronous orbit shown above is based on the rotational period of 12.8 ± 1.7 h obtained from occultation data by Elliot *et al.*¹⁰.

the break-up of the original satellite. The positions of the rings with respect to the Roche zones and the synchronous orbits suggest that the jovian ring satellites have densities $\geq 3 \text{ g cm}^{-3}$ and that the uranian ring satellites have densities $\geq 2 \text{ g cm}^{-3}$. Most of Saturn's broad ring system and the newly discovered narrow rings lie above its synchronous orbit, hence the mechanism of ring formation discussed above cannot be applied.

Particle paths

The path of a ring particle when the orbit of the ring satellite is circular can be studied by considering the Jacobi integral. For motion in horseshoe orbits it is convenient to write the Jacobi constant C as¹¹

$$C = 3 + \alpha m^{2/3} \quad (1)$$

where m is the satellite-planet mass ratio. We have found, by numerical integration of particular cases, that the type of path followed by a particle depends on the value of α . If we write

$$a_i = 1 + \Delta a_i \quad (i = 0, 1, 2) \quad (2)$$

and

$$|\Delta a_0| - |\Delta a_j| = \pm m^n \quad (j = 1, 2) \quad (3)$$

where a is the semimajor axis of the particle and the subscripts refer to the number of consecutive encounters with the ring satellite that the particle has experienced, then we find that n varies with α and that n increases to values near unity as α tends to zero. Our results for $m = 10^{-9}$ are shown in Fig. 4. We also conclude from these experiments that motion in a horseshoe is

unlikely if α exceeds about 1.2. The existence of this limit may account for the sharp edges of the rings.

For motion in a circle

$$\Delta a = 2 \left(\frac{\alpha}{3} \right)^{1/2} m^{1/3} \quad (4)$$

Thus the width of the ring is proportional to $m^{1/3}$ and a wide range of satellite masses produces a comparatively narrow range of ring widths. This could account for the observation that the uranian rings have a small spread of widths.

For small values of α (≤ 0.3), the changes in a on encounter are such that Δa_0 and Δa_1 are almost exactly equal and opposite. If we write

$$\delta a = (|\Delta a_0| - |\Delta a_1|) / \Delta a_0 \quad (5)$$

then

$$\delta a \sim \pm m^{n-1/3} \quad (6)$$

and, for $\alpha \leq 0.3$, $n \geq 2/3$ and $|\delta a| \sim m^{1/3}$. Thus, for $m \leq 10^{-9}$ (10^{-9} is greater than the satellite masses needed to account for the widths of the uranian rings) $|\delta a| \leq 10^{-3}$ and

$$\Delta a_0 = -\Delta a_1 \quad (7)$$

to 1 part in 10^3 or greater (see Fig. 5). It is this symmetry which accounts for the peculiar stability of very narrow rings.

This symmetry is not a property of the circular orbit case alone. If the ring satellite has an eccentric orbit, and if Δa_0 or α is small and e_0 and e_s , where subscript s refers to the ring-satellite, are not too different, then the changes in a on encounter are still symmetrical. Here, we define α by equation (4) even though the Jacobi integral does not apply to the eccentric orbit case. Our results for $m = 10^{-9}$ and $e_s = 0.01$ (which is close to the mean eccentricity of the uranian ϵ ring) are shown in Fig. 4. There seems to be no substantial difference between the circular orbit and the eccentric orbit cases.

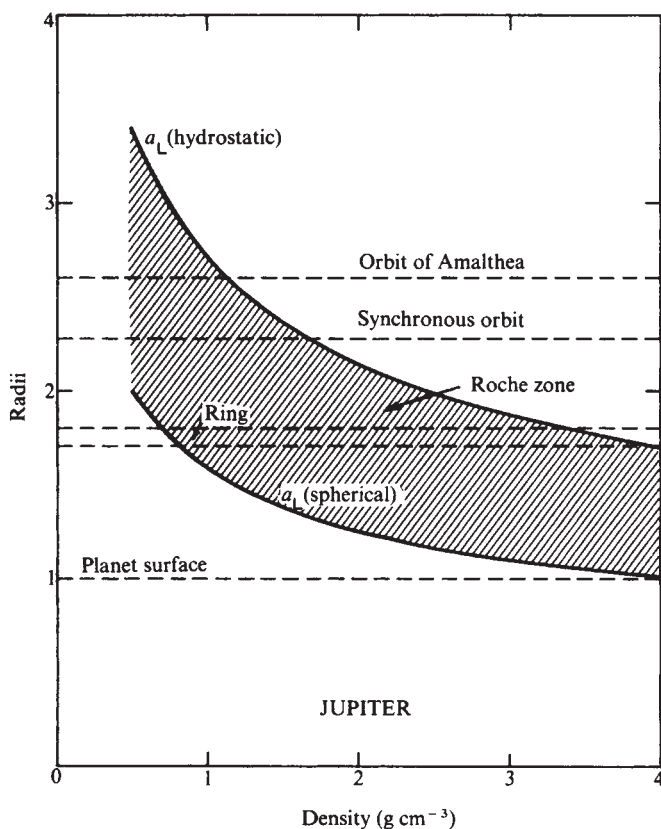


Fig. 3 Roche limit for Jupiter as a function of satellite density. Note the similarity between the location of the rings on this figure and those in Fig. 2.

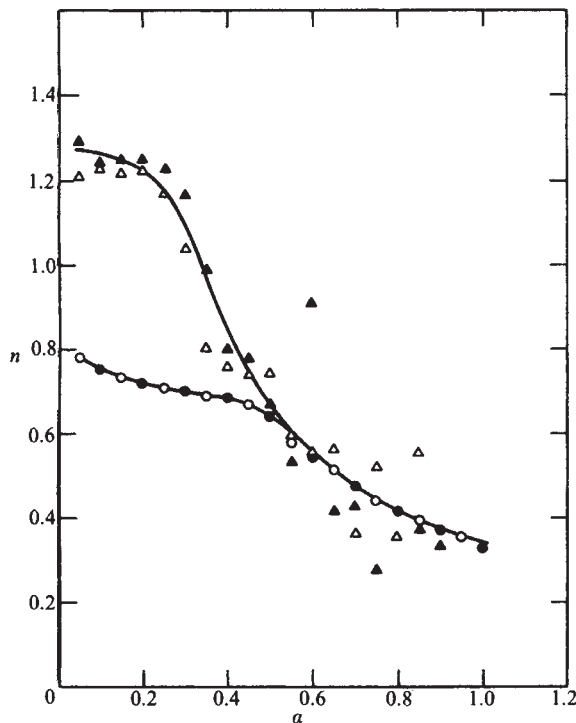


Fig. 4 n is a measure of the symmetry of the horseshoe path about the line $a = 1$ (see equation (3) and Fig. 5). The circular points refer to changes in the semimajor axis a of the ring particle orbit after a single encounter with the ring-satellite: ●, the circular orbit case ($e_s = 0$, where e_s is the eccentricity of the ring-satellite orbit); ○, the elliptical orbit case ($e_s = 0.01$). Triangles indicate the total change in a after two consecutive encounters: ▲, the circular orbit case ($e_s = 0$); △, the elliptical orbit case ($e_s = 0.01$).

The effect on the orbit of a ring particle of an external force due, for example, to Poynting–Robertson light drag is shown in Fig. 5. If α is small (≤ 0.3), then Δa_0 and Δa_1 are always equal and opposite: drag forces have little influence on this phenomenon. Therefore, if the ring is very narrow and the magnitudes of the drag forces acting on the particle on the inside and on the outside of its horseshoe path are not too different, then the orbital decay achieved in one half of the path is, in effect, cancelled by that achieved in the other. Second order effects due to the finite width of the ring are very small and we will not discuss them here.

As the drag force extracts angular momentum from the ring system, some orbital decay must occur. But the orbits of the ring particles and the ring satellite decay together at some rate r times less than that of an unconstrained ring particle.

$$r \sim \frac{m_r}{m + m_r} \quad (8)$$

where m_r is the total mass of the ring particles. For a ring of optical depth unity,

$$r \sim 5 \times 10^{-4} \left(\frac{a}{50,000 \text{ km}} \right) \left(\frac{d}{0.1 \text{ cm}} \right) \left(\frac{10 \text{ km}}{W} \right)^2 \quad (9)$$

where W is the mean width of the ring. Thus, even if the rings of Uranus consist of very small particles ($d \ll 0.1 \text{ cm}$), Poynting–Robertson drag acting over times comparable with the age of the Solar System would not result in significant orbital decay or ring spreading.

Radiation pressure acting on small (micrometre size) particles in an optically thin ring could distort the particle paths and, in the case of the jovian ring, the discussion we have given in terms of simple horseshoes may not be appropriate for these very small particles (J. A. Burns, personal communication). However, we contend that the presence of small satellites in the jovian ring is needed not only to provide a localised source of ring material,

but also to account for the partial confinement of the ring particles in a comparatively narrow lane.

Ring geometry

The remarkable variation in width of the uranian ϵ ring is a consequence of the ring's large eccentricity gradient $g (= a \, de/da)$. The ratio w of the maximum and minimum ring widths is given by¹

$$w = \frac{1+g}{1-g} \quad (10)$$

The mean ring width is $2\Delta a$, where Δa is half the difference of the semimajor axes of the ring's inner and outer edges. Since w must be positive, we must have $g < 1$. For the uranian ϵ ring $\Delta a = 30 \text{ km}$ and $g = 0.62$ (ref. 12). Thus, g is not merely non-zero, it is also close to the critical value of unity. Accounting for this phenomenon is by far the largest hurdle that any theory of the rings of Uranus has to surmount.

If a ring has a uniform eccentricity gradient and the particles in the ring have a common pericentre, then the relative velocities at pericentre are very much less than those at apocentre. We consider that it is probably this asymmetry which gives rise to the systematic changes in the orbital elements of the ring particles on encounter with the ring satellite.

Some actual changes in the orbital elements produced by encounter are shown in Fig. 6. The parameters chosen for this particular numerical experiment were $\alpha = 1.2$, which is the critical value of α for horseshoe motion, $m = 10^{-10}$ and $e_s = 0.00780$, which is the mean eccentricity of the uranian ϵ ring. If $a = 51,284 \text{ km}$, which is the mean semimajor axis of the uranian ϵ ring, then these parameters give $W = 2\Delta a = 60 \text{ km}$, which is the observed mean width of the ring. The values of a_s , e_s , a_0 and e_0 were dictated by the observed eccentricity and eccentricity gradient of the ϵ ring. Thus, particles on the outside of the ring were given values $e_0 = 0.00816$ and those on the inside values $e_0 = 0.00744$. The initial phases of the particles with respect to the satellite were chosen randomly with approximately equal numbers on the inside and on the outside of the ring.

The histogram of the values of g produced by this experiment (see Fig. 6) shows a strong positive bias with a peak at $g \approx 0.5$, which is close to the gradient ($g = 0.62$) defined by the edges of the ϵ ring. However, many factors will have an influence on this experiment. These include α , m , and, in particular, the shape of the satellite. These factors have yet to be investigated in detail. It would appear that the horseshoe orbit model of Dermott *et al.*¹ may account for the observed eccentricity gradient of the uranian ϵ ring, but we do not regard this question as settled. However, we have confirmed that the changes in pericentre $\tilde{\omega}$ of the ring particle orbit on encounter are small ($\leq 2^\circ$). Hence, as particles in horseshoe orbits spend equal amounts of time on the

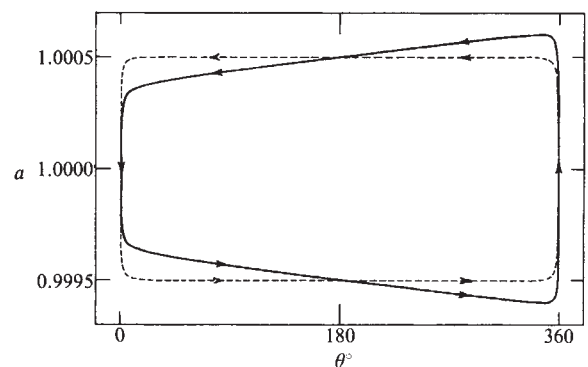


Fig. 5 Path of a particle moving in a horseshoe orbit about a ring-satellite of mass $m = 10^{-9}$ (circular orbit case). Semimajor axis a is plotted against the angular separation θ of the particle and the satellite. The dashed line refers to the particle path in the absence of drag and the solid line shows the effect of an external drag force. Both paths are symmetrical about the line $a = 1$.

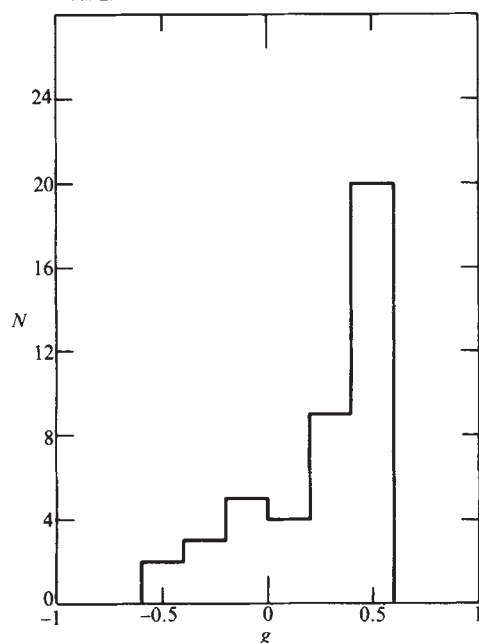


Fig. 6 Histogram showing the changes in eccentricities and semimajor axes produced by encounter with a ring-satellite in an eccentric orbit ($e_s = 0.00780$). The initial phases of the particles with respect to the satellite were chosen randomly. The strong positive bias in the distribution of $g = (e_0 - e_1)/(a_0 - a_1)$, where the subscripts 0 and 1 refer to values before and after encounter) could account for the observed eccentricity gradient ($= 0.62$) and hence the variation in width of the uranian ϵ ring.

inside and the outside of the ring, their average precession rate equals that of the ring-satellite and differential precession associated with the oblateness of the planet does not result in ring spreading. The pericentres of the ring particles remain aligned.

Stability

Even in the absence of drag, the particle path shown in Fig. 5 is not closed, since after every second encounter of a particle and the ring-satellite $|\Delta a|$ has changed in total by some small amount $\sim \pm m^n$ (see equation (3)). This suggests that the evolution of $|\Delta a|$ can be described by a random walk process and, since $n \leq 1$, that a particle will be lost from the ring after $\approx (1/2 W/m)^2$ encounters. The time between encounters is $2/3 \Delta a^{-1}$. From equation (4) we have

$$1/2 W \approx m^{1/3} \quad (11)$$

Hence, the lifetime of a ring particle is

$$\Gamma \approx \frac{T}{m^{5/3}} \quad (12)$$

where T is the orbital period of the ring satellite in arbitrary units.

Paradoxically, Γ increases as the mass of the ring satellite decreases: very narrow rings are particularly stable. For the Jupiter-Sun system $m \sim 10^{-3}$ and $T \approx 12$ yr; hence $\Gamma \sim 10^6$ yr and the lifetime of a ring orbiting the Sun with Jupiter would be short. It is probably significant that none of the Trojan asteroids are observed to have horseshoe paths. For a planetary ring, $T \sim 10^{-3}$ yr and if $m < 10^{-9}$, and this is greater than the masses needed to account for the widths of the uranian rings, then $\Gamma > 10^{12}$ yr.

Γ is not simply the lifetime of the ring, it must also be the time needed by a planet or satellite to sweep up small particles from its own orbit. If $T \sim 10^{-3}$ yr and $m < 2 \times 10^{-8}$, then $\Gamma > 5 \times 10^9$ yr. This suggests that very small satellites in the Solar System, which have not been captured and which lie outside the Roche zone, may be associated with narrow rings of primordial material that they have yet to accrete. We suggest that this could account for the newly discovered rings of Saturn. A ring of width ≤ 500 km

and radius 140,000 km would need only a satellite of mass $\leq 6 \times 10^{-9}$ (and diameter ≤ 200 km) to stabilise it. Such a ring would have a lifetime $\Gamma \geq 5 \times 10^{10}$ yr.

Discussion

The location of narrow rings and satellites just inside the Mimas first-order resonances supports the suggestion of Goldreich and Tremaine^{7,13} that these locations could be sites of preferential satellite formation. The observations are consistent with their theory that Mimas excited trailing spiral density waves in a disk of particles surrounding the planet at the positions of the $(p+1):p$ resonances (p is an integer) and that this resulted in the transfer of material towards the planet. Inside the Roche zone, this transfer of material has probably produced Cassini's and other divisions, the inner edges of which are associated with first-order resonances^{7,13}. We suggest here that outside the Roche zone this mechanism has resulted in satellite formation and that these small satellites maintain rings of particles they have yet to accrete.

Van Allen *et al.*⁵ claim to have detected possibly two satellites in the F ring and several small satellites outside this ring. Yet the probability of detecting a single, small satellite by their method is as low as $\sim 1\%$. In the 'ring' which they initially named the G-ring (2.490 to 2.550 R_s), both of these supposed satellites have diameters ≥ 170 km, but the separation of their orbits is only ~ 720 km (ref. 5). We suggest that their results could equally be accounted for by an eccentric ring of particles of width ≥ 170 km which is maintained by a single satellite of diameter ~ 45 km moving in an orbit of eccentricity ~ 0.005 . Similarly, the structure observed in the F-ring profile, which could be due to two satellites whose orbits are separated by only 420 km, could equally be accounted for by a ring of particles maintained by a single satellite moving in an orbit of eccentricity ~ 0.003 . Alternatively, the F and G rings could consist of a number of discrete narrow rings maintained by an equal number of small satellites. Both of these suggestions are less improbable than the suggestion of Van Allen *et al.*⁵ that on five separate occasions the Pioneer 11 spacecraft passed within a few degrees of the longitude of a small satellite.

Finally, if small satellites do exist inside the outer edges of the F and G rings, then the fact that the present orbit of Mimas is associated with the outer edges of these rings could place severe limits on changes in Mimas' orbit since the time of satellite formation. This conclusion could have profound implications with respect to the origin of resonances and the formation of satellites in the Solar System and the long-term stability of satellite orbits.

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Note added in proof: This article was written before the publication of the Pioneer 11 results in *Science*, 25 January 1980, and at the time we were not aware of the results of J. A. Simpson *et al.*¹⁴. They report that the distribution of matter in the F-ring is markedly non-uniform: the electron absorption features exhibit large radial and azimuthal variations. This supports our suggestion that the F-ring contains a number of small satellites, moving in elliptical orbits, which maintain narrow rings of particles of non-uniform width. They also report the detection of material distant 3.07 R_s from Saturn and close to the orbit of Mimas. But since the spacecraft was actually 0.04 R_s outside the orbit of Mimas at the time of the observation, we conclude that this material cannot be part of a narrow ring of the type we have described. However, since Mimas was $\sim 50^\circ$ ahead of the spacecraft at the time, the observation may be accounted for if Mimas, like Jupiter, has a number of small bodies moving in tadpole orbits about its equilateral lagrangian equilibrium points. These bodies must also be in near-resonance with Tethys, hence their orbits probably have appreciable forced eccentricities.

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Origin of rare gases in the Earth

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Although terrestrial rare gases have elemental abundances similar to those of meteorites (planetary-type rare gases), their Ne and Xe isotopic compositions are quite different which suggests that degassing of meteoritic materials alone cannot explain the atmospheric rare gases. The terrestrial rare gases must contain heavily fractionated rare gases captured by planetesimals during a fragmentation process (planetesimal-type rare gases) as well as the planetary type rare gases.

THE approximate similarity in elemental abundance pattern between the Earth and meteorites has led many investigators to assume^{1,2} that terrestrial rare gases were derived from those trapped by chondritic materials, that is, the planetary-type rare gases. However, the isotopic compositions of the atmospheric Xe and Ne are significantly different from those in the chondrites³. It is clearly not possible to derive the terrestrial Xe and Ne simply from the chondrite rare gases and the explanation for the difference should be sought in the pre-planet conditions rather than in the particular evolution processes in the Earth or in the chondrites after their formation³. Astrophysical considerations also indicate that the grains condensed from the solar nebula which subsequently formed the Earth had mostly existed around the present Earth orbit⁴, whereas chondrite parent bodies are generally regarded to have formed further from the Sun, most likely near the asteroidal belt (~ 2 – 4 AU) (refs 5, 6). Because the physical conditions in the solar nebula such as temperature and pressure must be different between the asteroidal belt and the Earth orbit, there seems no compelling reason to suppose that

the original Earth materials had trapped the rare gases in the same way as did the chondritic meteorites or their parent bodies. For example, the low temperature adsorption hypothesis⁷ which was proposed to explain the chondritic rare gas pattern is not applicable to the Earth, as the nebula temperature around 1 AU was too high, ~ 225 K (ref. 8) for grains to adsorb any significant amount of rare gases. This consideration does not, however, rule out the possibility that some of the terrestrial rare gases were supplied from the chondrites, as part of the chondritic materials must have been brought to the Earth's region by the gas drag force and mixed with the materials which formed the Earth⁹. In fact, chondritic materials must be called for to explain the material balance, particularly that of the volatile elements in the Earth^{10,11}. The past decade has seen considerable progress in astrophysics, such as the discovery of the fragmentation process^{12–14} and the subsequent formation of planetesimals. We show here that astrophysical theory leads to a conclusion that a substantial amount of gravitationally-fractionated rare gases must have been captured by planetesimal which later formed the Earth. Hence, the terrestrial rare gases consist of both these planetesimal type rare gases and those trapped in the chondritic materials, that is, the planetary type rare gases. This mixing model can explain the large isotopic fractionation of the terrestrial Xe relative to the planetary and solar type Xe. Except for the Kr and Ar ratio, the model is also consistent with other characteristics of the terrestrial rare gases.

Constraints on terrestrial rare gases

The characteristic features of the rare gases in the Earth which any theory should explain are summarised below. (For the detailed compilation of the terrestrial rare gas characteristics outlined here, see ref. 3.)

(1) The rare gases are depleted relative to the solar abundance. The depletion ranges from 10^{-6} (Xe) to 10^{-11} (Ne). The elemental abundance pattern is similar to that of the planetary type rare gases except for Xe, the latter being more depleted in the Earth.

(2) The isotopic composition of terrestrial Xe shows large mass-dependent fractionation relative to the planetary Xe, in that the heavier isotopes are more enriched (about 4% per AMU). The pattern is surprisingly linear with mass number for all but ^{129}Xe and the heaviest isotopes (see Fig. 2). Departure from the linear fractionation at the heavy end is usually attributed to fission effects, and at ^{129}Xe to extinct radioactive ^{129}I .

(3) Ne isotopic abundance is different both from the solar and planetary Ne (Ne B and Ne A respectively), in that the terrestrial

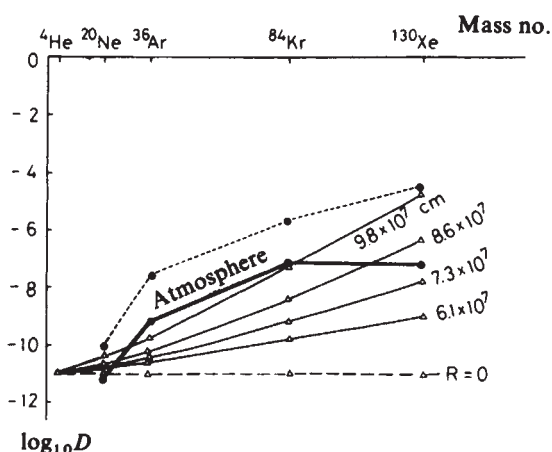


Fig. 1 Rare gas elemental abundance ($\log_{10} D$) relative to the solar abundance in planetesimals of various sizes (R) are plotted against a mass number. The amount of rare gases is normalised to Si. In the case of the atmospheric values (thick line), M_i stands for the rare gases in the atmosphere and Si in the whole Earth. Note that the mixing of the gravitationally fractionated planetesimal type (thin lines) and the planetary type rare gases (dotted line) would approximate the atmospheric pattern. [$D = (M_i/\text{Si})_{\text{atm}} / (M/\text{Si})_{\text{solar}}$].

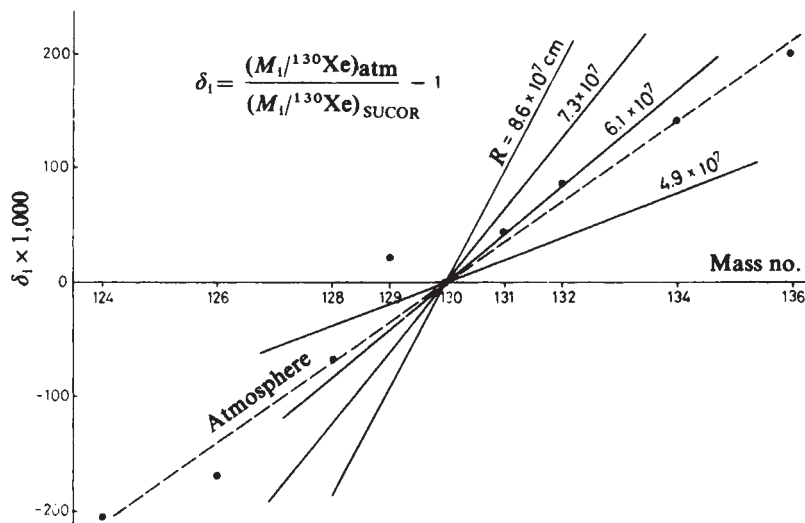


Fig. 2 Isotopic fractionation pattern for Xe in planetesimals of various sizes relative to SUCOR Xe (ref. 3) (SUCOR designates surface-correlated gas in lunar soil, which is regarded to be close to the solar composition). Lines indicate the fractionation for a planetesimal with a radius R (cm), which resulted from the gravitational fractionation in the planetesimal.

Ne lies roughly midway between the two types in a $^{20}\text{Ne}/^{22}\text{Ne}$ – $^{21}\text{Ne}/^{22}\text{Ne}$ diagram.

(4) In spite of the large difference in Ne and Xe isotopic compositions between the Earth and chondrites, Ar, and Kr seem to exhibit nearly the same isotopic compositions, although terrestrial Kr shows slight enrichment in the lighter isotopes of $\sim 0.5\%$ per AMU. However, the constraints (3) and (4) may not be as stringent as the constraints (1) and (2), because of uncertainties in defining the representative planetary Ne and Kr isotopic ratios.

Planetesimal type rare gases

There are numerous astrophysical theories of the origin of the Solar System and the formation of the planets. Here, we follow the astrophysical theories developed by Hayashi and his colleagues^{13,15–17}. According to these authors, grains condensing from the solar nebula gradually sedimented to the median plane. When the sedimented grain density exceeds a critical value ($\sim 10^{-7} \text{ g cm}^{-3}$), the grains would suddenly collapse to form numerous planetesimals, of size about 10^{18} g . This process is called fragmentation of the dusty layer and was obtained from the astrophysical dynamics independently by Safronov¹², Hayashi¹³ and Goldreich and Ward¹⁴. As the fragmentation occurred $\sim 10^4$ – 10^5 yr after the formation of the proto-Sun¹⁶, there still existed solar nebula gas during the fragmentation. The planetesimals thus formed were loosely-compacted grain ensembles, in that the central pressures were ~ 1 – 10^2 atm for planetesimals with radii 10^6 – 10^7 cm respectively¹⁷. Hence, there must have been considerable inter-grain free space or porosity, which was filled with the solar nebula. The time constant for the heavier rare gases to reach gravitational equilibrium within a planetesimal of a radius R (cm) is of the order of $R^2/(4 \times 10^{10}) \text{ yr}$ (R in cm), whereas the growth time for a planetesimal is $\sim 10^4 \text{ yr}$ (ref. 18). Hence, the rare gases in a planetesimal with a radius less than a few hundred kilometres are in gravitational diffusion equilibrium. When a planetesimal grows further, the gases are no longer in equilibrium and are 'frozen' in the last equilibrium state, corresponding to a planetesimal with a radius of a few hundred kilometres. The rare gases thus frozen in the planetesimals were finally incorporated in the terrestrial planets.

For rare gases in gravitational equilibrium within the planetesimals the density distribution can be expressed as

$$\frac{1}{\rho_i} \frac{dP_i(r)}{dr} = -\frac{GM(r)}{r^2} \quad (1)$$

$$M(r) = \frac{4}{3} \pi r^3 \rho_s$$

where ρ_i , ρ_s are the densities of the rare gas i and the planetesimal solids (assumed to be constant), P_i the pressure of the rare

gas i , G the gravitational constant, M the mass of the planetesimal, r the distance from the centre of the planetesimal. Assuming that the rare gases follow the equation of state for an ideal gas and that the temperature is constant, equation (1) yields:

$$\rho_i(r) = \rho_i(R) \exp(A\mu_i(1-x^2))$$

$$A = \frac{H}{kT} \frac{2\pi}{3} \rho_s G R^2$$

$$x \equiv r/R$$

where k is Boltzmann's constant, T the absolute temperature, μ_i the atomic weight of the rare gas i , H the mass of hydrogen atom, R the radius of the planetesimal and $\rho_i(R)$ is assumed to be equal to the density in the surrounding nebula at the surface of the planetesimal ($r = R$). Hence, assuming a constant porosity s for various size of planetesimals, the total mass M_i of the rare gas in the planetesimal is written as,

$$M_i = 4\pi \int_0^R r^2 \rho_i dr \quad (2)$$

$$= 4\pi R^3 s \rho_i(R) \exp(A\mu_i) \int_0^1 x^2 \exp(-A\mu_i x^2) dx$$

We then define a retention factor iD

$${}^iD \equiv (M_i/M_{Si})_p / (M_i/M_{Si})_{\text{solar}}$$

where Si denotes a conventionally chosen standard element Si and the subscript p refers to a planetesimal. Considering that the mass ratio of the solid phase to the gas phase in the condensing solar nebula is 0.00343 (ref. 19), the retention factor iD can be expressed with the aid of equation (2),

$${}^iD = 0.00343 \times s \rho_g / \rho_s$$

$$\times 3 \exp(A\mu_i) \int_0^1 x^2 \exp(-A\mu_i x^2) dx \quad (3)$$

or

$${}^iD \equiv ({}^iD)_0 \alpha_i$$

$$({}^iD)_0 \equiv 0.00343 \times s \rho_g / \rho_s$$

$$\alpha_i \equiv 3 \exp(A\mu_i) \int_0^1 x^2 \exp(-A\mu_i x^2) dx$$

where $({}^iD)_0$ corresponds to the retention factor for the planetesimal with a negligible gravitation ($A \rightarrow 0$), ρ_g nebula gas pressure and α_i indicates the enrichment factor of the rare gas i due to the gravitational force of the planetesimal.

In Table 1, we show the values of α_i for planetesimals of various sizes. To calculate α_i , we took $\rho_s = 2$ (in cgs) and $T = 225 \text{ K}$, the values being estimated for the solar nebula at 1 AU

Table 1 Enrichment factor α_i for rare gas i in planetesimals with a various radius R

$R(\times 10^7 \text{ cm})$	α_i				
	^4He	^{20}Ne	^{36}Ar	^{84}Kr	^{130}Xe
0.12	1.00	1.00	1.00	1.00	1.00
1.2	1.00	1.02	1.03	1.08	1.13
6.1	1.10	1.64	2.61	14.3	99.5
7.3	1.18	2.10	4.34	67.3	1.46×10^3
8.6	1.20	2.87	8.48	500	4.1×10^4
12	1.47	12.2	190	2.9×10^6	8.7×10^{10}

(ref. 8). For the smaller planetesimals ($R \leq 10^6 \text{ cm}$) the enrichment is negligible, whereas a planetesimal with $R = 8 \times 10^7 \text{ cm}$, Xe enrichment amounts to more than 10^4 .

Figure 1 displays the relative elemental abundances of the planetesimal type rare gases normalised to the solar abundances, for which we used a value of $\rho_s = 3 \times 10^{-8}$ (in cgs) at 1 AU (ref. 8) and $S = 0.33$ deduced as the ratio of $(\rho - \rho_s)$ to the density of crystalline silicate ($\rho \approx 3$). The horizontal line (dashed line) represents the case $A = 0$ (no gravitational field), for which $\sim 10^{-11}$ depletion occurs in all rare gases, corresponding to the ratio of the solid to the gas phases in the planetesimal. Figure 1 also shows the rare gas abundance patterns in both the terrestrial atmosphere (thick line) and the planetary type rare gases (dotted line) relative to the solar abundance. Although the planetesimal type rare gases can roughly account for the elemental abundance, the pattern is not exactly the same as that observed in the atmosphere. As discussed above, we must also take into account the planetary type rare gases which were introduced by chondritic materials in the terrestrial atmosphere. A mixing of the two types would better approximate the atmospheric rare gas abundance pattern.

Rare gas isotopic fractionations in planetesimals

The gravitational mass fractionation mechanism also leads to an isotopic fractionation. Isotopic fractionation is conveniently expressed in terms of δ_i -values relative to the solar isotopic composition. In the case of Xe δ_i will be defined conventionally as

$$\delta_i = (^i\text{Xe}/^{130}\text{Xe})/(^i\text{Xe}/^{130}\text{Xe})_{\text{solar}} - 1$$

δ_i is then expressed in terms of the retention factor iD , and thus of α_i defined in equation (3),

$$\delta_i = (D_i)/(D_{^{130}\text{Xe}}) - 1 = (\alpha_i)/(\alpha_{^{130}\text{Xe}}) - 1 \quad (4)$$

As $\mu_i \gg 1$ and $\mu_i \gg \Delta_i \equiv |\mu_i - 130|$ for the Xe isotopes, equation (4) can be approximately expressed as

$$\delta_i \approx \left\{ A - \frac{3}{2} \frac{1}{130} \left(1 - \frac{1}{\alpha_i} \right) \right\} \Delta_i$$

Hence, the gravitational mass fractionation indicates a linear mass fractionation trend in agreement with the observation. Table 2 shows the isotopic fractionation δ_i per atomic mass unit for various A , that is, for the planetesimals with various radius R . The isotopic mass fractionation is larger for the heavier rare gases and for the larger planetesimals.

Table 2 Isotopic fractionation factor δ_i (see text for the definition) for rare gas i in planetesimals with various radius R

$R(\times 10^7 \text{ cm})$	δ_i			
	^{20}Ne	^{36}Ar	^{84}Kr	^{130}Xe
1.2	$< 10^{-4}$	$< 10^{-3}$	$< 10^{-3}$	$< 10^{-3}$
6.1	$< 10^{-2}$	0.01	0.02	0.03
7.3	0.01	0.02	0.03	0.04
8.6	0.02	0.04	0.06	0.07
12	0.16	0.18	0.20	0.21

For planetesimals larger than $8 \times 10^7 \text{ cm}$, Xe isotopic fractionation amounts to $\sim 7\%$ per AMU. The δ_i values or a mass fractionation line calculated for various size of planetesimals are shown in Fig. 2 together with the case for the atmospheric Xe. Figure 2 shows that the atmospheric Xe mass fractionation trend can be explained as a mixture of fractionated planetesimal type Xe with the planetary type Xe which shows little mass fractionation relative to the solar isotopic ratio. Owing to the small mass number, the planetesimal Ne isotope shows little mass fractionation relative to the solar type Ne. Hence, we expect from the mixing model that the terrestrial Ne has the isotopic composition somewhere between the planetary and the solar type rare gases; this is, in fact observed in the terrestrial Ne (ref. 3). Because of the particular shapes of the retention factor D for the planetesimal and the chondrite (Fig. 1), that is, upward concave for the former and downward concave for the latter, the planetesimal type Ar and Kr could be less conspicuous in a mixture in which planetesimal gas is the major contributor of Ne and Xe. This may account for the approximate similarity in the Ar and Kr between the planetesimal and planetary-type rare gases, while Xe and Ne isotopic compositions are significantly different from each other.

Origin of the rare gases in the Earth

We first adopt the generally accepted assumption that the terrestrial atmosphere is of a secondary origin, in that the atmosphere was degassed from the interior of the Earth²⁰. We also assume that the atmospheric rare gases essentially represent the rare gases in the whole Earth.

We conclude that the terrestrial rare gases can be interpreted as a mixture of planetary type rare gases which were added to the Earth by chondritic materials and the planetesimal type rare gases captured by planetesimals which later accreted the Earth. As we do not know the exact size distribution of the planetesimals which later accreted the Earth, it would be meaningless to attempt a quantitative estimation of the degree of mixing between the two types of the rare gases. Here we would only suggest that mixing of roughly equal amounts of the planetary- and planetesimal-type rare gases can explain some of the observed characteristic of the terrestrial rare gases: (1) the enormous depletion of the rare gases relative to the solar abundance, with the light rare gases more depleted; (2) the large Xe isotopic fractionation ($\sim 4\%$ per AMU) with nearly a linear mass dependence; (3) the difference in Ne isotopic compositions among terrestrial, planetary, and solar type rare gases. The situation here—previously seen as a mixing of the two latter to make the first—has not been affected by our model because the planetesimal modifications of the isotopic composition of neon should be small. On the other hand, difficulties for the model are the isotopic uniformities everywhere for argon and krypton because we would expect the planetesimal effects on argon, and especially krypton, to introduce effects which are not, in fact seen. Also, the model does not offer a direct explanation of the Xe deficiency in the atmosphere relative to the planetary type rare gases. We suggest that the deficiency may be due to the difficulty in its degassing from the Earth.

For the origin of the planetary type rare gases, we offer no specific explanation; it could be low temperature adsorption⁷, or crystal growth trapping²¹ or others^{22–24}, but the specific mechanism for the origin is not important in the present discussion. Finally, the mixture model should also apply to other inner planets. We therefore suggest that Xe and Ne isotopes should show similar isotopic fractionation trends relative to the solar type isotopic compositions to those observed in the terrestrial rare gases.

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Synthesis in *E. coli* of a polypeptide with human leukocyte interferon activity

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Double-stranded cDNA prepared from the 12S fraction of poly(A) RNA from interferon (IF)-producing human leukocytes was cloned in Escherichia coli using the pBR322 vector. One of the resulting clones had a 910-base pair insert which could hybridise to IF mRNA and was responsible for the production of a polypeptide with biological IF activity. Up to 10,000 units IF activity per g of cells was obtained from some clones.

CELLS of almost all vertebrates, when exposed to certain viruses or inducers, produce one or more (glyco)proteins, known as interferons^{1,2}. Interferons (IFs) are characterised biologically by their ability to induce in target cells a virus-resistant state which is associated with the *de novo* synthesis of several proteins, in particular a protein kinase³, an oligoadenylate synthetase^{4,5} and a phosphodiesterase⁶. In addition, IFs have a regulatory effect on the immune response^{7,8} and their enhancement of

killer lymphocyte activity⁹ may be the basis of their inhibitory effect on tumour growth².

Two major classes of acid-stable (type I) IFs have been recognised in man—leukocyte interferon (Le-IF), released by stimulated leukocytes, and fibroblast interferon (F-IF), produced by stimulated fibroblasts. Le-IF and F-IF differ not only immunologically but also in their target cell specificity: whereas both IFs induce a virus-resistant state in human cells, Le-IF is also very active on bovine, porcine and feline cells, whereas F-IF is not². The two IFs are encoded by separate mRNAs¹⁰.

Human Le-IF has been purified more than 80,000-fold, to a specific activity of 4×10^8 units per mg (ref. 11) or 2.5×10^8 units per mg (ref. 12). Two components have been characterised by polyacrylamide gel electrophoresis, with apparent molecular weights (MWs) of 21–22,000 and 15–18,000, respectively^{13,14}; they are believed to differ in their degree of glycosylation¹⁴. Enzymatic¹⁵ or chemical¹⁶ removal of most or all of the carbohydrate moiety seems to have little effect on the biological activity of IF.

We sought to clone human Le-IF cDNA in order to construct bacterial strains producing polypeptide(s) with human IF activity, and to generate the tools required for the analysis of Le-IF gene structure and function. The particular difficulties of this undertaking were the lack of a purified Le-IF mRNA and our ignorance of the structure of Le-IF, which precluded the preparation of pure or highly enriched IF cDNA, or of a probe for the identification of the desired clones.

We describe here the isolation of a hybrid plasmid containing a 872-base pair Le-IF cDNA, which elicits the formation in *Escherichia coli*, of a polypeptide with the immunological and biological properties of human Le-IF.

Isolation of hybrid plasmids containing IF cDNA sequences

Hybrid DNA, consisting of leukocyte cDNA sequences joined to pBR322 at the *Pst*I site by means of dG:dC sequences, was prepared by conventional means, using as starting material a 12S fraction of poly(A) RNA from IF-producing leukocytes, purified about 10-fold for IF mRNA. cDNA cloned in this fashion is usually flanked by *Pst*I sites and, as it is located in the

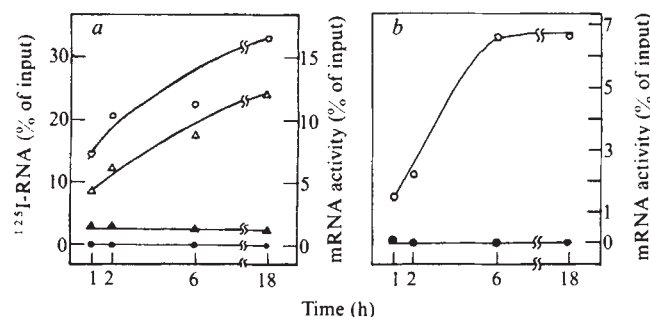


Fig. 1 Hybridisation of IF mRNA and ¹²⁵I-globin mRNA to filter-bound DNAs. DNA was linked to DPT paper as described elsewhere¹⁶. *a*, For each time point, one 0.25-cm² piece of DPT paper with 500 ng of *Hind*III-excised insert of rabbit β -globin cDNA plasmid and one piece with 700 ng of *Hind*III-digested pBR322 were hybridised as a sandwich with 10 μ l of hybridisation medium²² containing 200 ng ¹²⁵I-labelled globin mRNA. *b*, As above except that DPT papers contained 250 ng of *Hind*III-excised insert of rabbit β -globin cDNA plasmid and 250 ng of *Pst*I-excised insert of Hif-2h, respectively, and hybridisation was with 5 μ g of Le poly(A) RNA in 10 μ l. In all cases hybridisation, washing and elution were as described in ref. 22. The RNA was recovered, its ¹²⁵I radioactivity determined, and injected into 40–50 oocytes. In experiment *b*, oocytes were incubated with 50 μ Ci ³H-histidine. Oocyte supernatants were assayed for IF activity by the cytopathic effect reduction assay (see Table 3 legend). Le poly(A) RNA (1 μ g) injected directly into 20 oocytes gave 2,700 units IF. For the determination of ³H-labelled globin formation, oocytes were homogenised, centrifuged and an aliquot of the supernatant electrophoresed through a 20% polyacrylamide gel³⁷. The globin band was cut out and the radioactivity determined in toluene-based scintillator solution. 100 ng globin mRNA injected directly gave 100,000 c.p.m. ³H-globin. *a*, ¹²⁵I-RNA hybridised to β -globin cDNA (○) or to pBR322 (●). ³H-labelled β -globin formed in oocytes after injection of RNA hybridised to β -globin cDNA or (Δ) pBR322 (▲). *b*, IF activity formed in oocytes after injection of RNA hybridised to Hif-2h fragment (○) or β -globin cDNA (●).

Table 1 mRNA hybridisation translation assay for the detection of IF cDNA in hybrid DNA from pools of transformed *E. coli*

DNA sample	Interferon activity
Expt 1: Pools of 512 clones	
I	<60 (<60); <u>110</u> (<20); <110 (<110); <110 (<110); <35 (<35)
δ	<u>20</u> (<20)
N	<u>35</u> (<20); <110 (<110); <u>200</u> (<110)
λ	<60 (<60); <u>60</u> (<20); <110 (<110); <110 (<110)
8 other groups negative	
Expt 2: Pools of 64 clones from sample λ	
λ -I	<35 (<35); <35 (<35)
λ -II	<u>130</u> (<30); <45 (<45)
λ -III	<u>225</u> (<35); <u>35</u> (<30); <u>35</u> (<30); <u>600</u> (<30); <20 (<20)
λ -IV	<u>85</u> (<35); <25 (<25)
λ -V to VIII	negative
Expt 3: Pools of 8 clones from sample λ -III	
λ -III-1	<20 (<20); <20 (60); <u>35</u> (<30)
λ -III-2	<35 (<35); <30 (<30); <u>150</u> (<20); <u>600</u> (<35); <u>110</u> (60)
λ -III-3	<25 (<25); <30 (<30)
λ -III-4	<u>30</u> (<30); <20 (<20); <20 (60)
λ -III-5 to 8	negative
Expt 4: Single clones from sample λ -III-4	
λ -III-4B	<35* (<35); <20 (60)
λ -III-4C	35 (60); <u>60*</u> (<35); <u>111*</u> (<11); <11 (<11); <u>20</u> (<20)

Hybrid DNA containing leukocyte cDNA was prepared as follows. To obtain poly(A) RNA from IF-producing leukocytes, 10^{11} human leukocytes were primed with Le-IF and induced with Sendai virus as described elsewhere²¹. After 5 h at 37°C the cells were collected, suspended in 1 l PBS and added to 17 l 20 mM Tris-HCl (pH 7.5), 1 mM EDTA and 2% SDS. The lysate was digested with Pronase (200 μ g ml⁻¹) for 1 h at 20°C. 2 M Tris-HCl buffer (pH 9, 5% vol) was added and the solution extracted with 15 l phenol for 30 min. Chloroform (3 l) was added to aid phase separation, the aqueous phase adjusted to 0.3 M NaOAc buffer (pH 5.5) and the nucleic acid precipitated with ethanol. The precipitate (about 1 g) was dissolved in 900 ml TNE [Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA] containing 0.5% SDS, extracted three times with phenol and exhaustively with ether, and the poly(A) RNA recovered by three batch adsorptions to 3 $\times$ 5 g oligo (dT) cellulose (type 7, P-L Biochemicals) followed by elution with water. The yield was 1.6 mg; 1 μ g gave rise to 300 units IF when injected into oocytes. For further purification, 860 μ g RNA in 5 mM EDTA were passed through a Chelex-100 column, heated for 90 s at 100°C and centrifuged through a 5–23% sucrose gradient in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.2 M NaCl. The fractions containing the IF-mRNA activity, sedimenting around 12S, were pooled and the poly(A) RNA recovered by oligo(dT) cellulose chromatography. The yield was 40 μ g; 1 μ g gave rise to 3,600 units IF when injected into oocytes. pBR322-linked Le-cDNA was prepared essentially as described previously²². Sucrose-gradient purified poly(A) RNA from two preparations (48 μ g) was used as template for reverse transcriptase to generate 10 μ g cDNA 600–1,000 nucleotides long; this was converted into double-stranded DNA by DNA polymerase I, and treated with S₁ endonuclease (yield, 8 μ g preparation A). Of this DNA, 5 μ g were centrifuged through a sucrose-density gradient, and material sedimenting faster than a 600-base pair ³²P-DNA marker was pooled and precipitated with ethanol (preparation B, 3 μ g). cDNA was elongated with dCMP residues, annealed to dGMP-elongated, PstI-cleaved pBR322 (ref. 17) and used to transform *E. coli* χ 1776 (ref. 23) (preparation A; 3.3×10^4 tetracycline-resistant transformants per μ g DNA), or *E. coli* HB101 (preparation B; 4×10^4 transformants per μ g DNA). Ten thousand colonies of transformed *E. coli* χ 1776 were inoculated individually into wells of microtitre plates and stored with 20% glycerol at –20°C. Five thousand colonies of transformed *E. coli* HB101 (from preparation A) were raised on Millipore filters and stored frozen as described by Hanahan and Meselson^{22,24}. To carry out the hybridisation translation assay, the number of bacterial clones (from preparation A) indicated in the table were inoculated individually on to agar plates, incubated for 24 h and washed off with medium. This suspension was used to inoculate 1-l cultures from which plasmid DNA was purified as described (method B in ref. 25). The hybrid Le cDNA (20 μ g) was cleaved with HindIII, mixed with 12 μ g Le poly(A) RNA, 5 ng ¹²⁵I-labelled rabbit β -globin mRNA (5,000 c.p.m.) and 0.1 μ g PstI-cleaved rabbit globin cDNA plasmid (Z-pBR322(H3)/Rc β G-4.13)²⁶ in 40 μ l 80% formamide, 0.4 M NaCl, 10 mM PIPES buffer (pH 6.4) and 5 mM EDTA, and heated for 4 h at 56°C. After diluting to 1 ml with 0.9 M NaCl and 0.09 M trisodium citrate (pH 7) and adjusting to 4% formamide, the solution was filtered through a Millipore filter (13 mm diameter, 0.4 μ m pore size). The filter was washed for 10 min at 37°C in 0.15 M NaCl, 0.015 M trisodium citrate and 0.5% SDS, and the RNA recovered by heating the filter for 5 min in 0.5 ml 1 mM EDTA, 0.5% SDS and 3 μ g ml⁻¹ yeast RNA at 75°C. The RNA was purified by oligo(dT) cellulose chromatography, precipitated with ethanol and assayed for IF-mRNA activity. To determine IF-mRNA activity, the RNA sample (up to 3 μ g) was dissolved in 1–3 μ l 15 mM Tris-HCl (pH 7.5) and 88 mM NaCl, and injected into 20–60 *Xenopus laevis* oocytes²⁷ (50 nl per oocyte). Oocytes were incubated for 12–16 h in Barth's medium²⁸, homogenised in 0.5 ml 50 mM Tris-glycine buffer (pH 8.9) and the supernatant was assayed for IF. In later experiments (Table 2), incubation was for 24–48 h and the incubation medium was assayed for excreted IF²⁹. IF was determined by the vesicular stomatitis virus (VSV) plaque reduction assay²¹. All values are expressed in international units. Values marked with an asterisk were obtained by hybridisation to diazobenzoyloxymethyl (DBM)-bound DNA, as described in ref. 22. Underlined values are considered positive. The control values (in parentheses) were obtained by hybridisation to pBR322 DNA. All manipulations involving live *E. coli* HB101 or *E. coli* χ 1776 containing Le cDNA-pBR322 hybrids were carried out in P3 containment conditions as described in the NIH Recombinant DNA Research Guidelines.

β -lactamase gene, can be expressed as a fused protein¹⁷ or, in certain circumstances, as an independent polypeptide¹⁸.

We identified an IF cDNA clone by a mRNA hybridisation translation assay¹⁹. Hybrid plasmid was prepared from pools of 512 bacterial clones, and 20 μ g of each plasmid pool were cleaved with PstI, denatured and annealed with 12 μ g crude Le poly(A) RNA. ¹²⁵I-Labelled globin mRNA and rabbit β -globin cDNA plasmid were added to monitor hybridisation and all subsequent steps. Hybridised RNA was recovered from the filters, purified and injected into oocytes to determine its IF-mRNA activity. Control hybridisations were carried out with pBR322. The overall recovery of β -globin mRNA activity was only about 5% of the input.

Four out of 12 groups of 512 clones (δ , λ , I and N) gave positive results by this assay, albeit erratically; controls were consistently negative in these groups (Table 1). However, in later experiments, controls occasionally gave a positive result, perhaps due to insufficient washing of the filters. A group of clones was scored as being positive if the value was higher than in the parallel control. The bacterial clones of group λ were arranged in 8 subgroups of 64 each, and assayed as above. Three of these subgroups, λ -II, λ -III and λ -IV, gave positive responses; the clones of λ -III were regrouped into eight sets of eight.

The set λ -III-4 was the first to yield a positive result; λ -III-2 subsequently also gave positive results. DNA was prepared from the single λ -III-4 clones and that from λ -III-4C gave positive responses both by liquid and filter-bound hybridisation (Table 1). After recloning in *E. coli* HB101 the hybrid plasmid of clone λ -III-4C, designated Z-pBR322(Pst)/HcIF-4c (abbreviated to Hif-4c), was purified and cleaved with PstI; it released a 320-base pair insert, that is, a fragment about one-third of the expected length of complete IF cDNA. The fragment bound IF mRNA efficiently (Table 2).

A set of colonies containing hybrid DNAs related to Hif-4c was identified by *in situ* hybridisation with ³²P-labelled Hif-4c PstI fragment. Among the 64 clones of λ -III, three gave a strong hybridisation response, namely 4C, 2H and 7D, and two (1E and 3D) a weak one. λ -III-2H had the largest insert, about 900 base pairs; it was recloned in *E. coli* HB101 and designated Z-pBR322(Pst)/HcIF-2h (abbreviated to Hif-2h). In addition, 5,000 clones prepared as described, but using double-stranded Le-IF cDNA selected for length above 600 base pairs (preparation B, cloned in *E. coli* HB101), were screened by *in situ* hybridisation, using the same probe. Of 185 positive clones identified, 95 gave a strong and 90 a weak hybridisation response in the Grunstein-Hogness assay²⁰. The former were

designated *E. coli* HB101(Z-pBR322(*Pst*)/HcIF-SN1 to -SN95) (abbreviated to SN1 to SN95).

Properties of plasmid Hif-2h

The insert of plasmid Hif-2h, released by *Pst*I cleavage, was attached to diazophenylthioether (DPT) paper and the kinetics of hybridisation to IF mRNA in conditions of DNA excess determined (Fig. 1). In optimal conditions, about 7% of the IF-mRNA activity and 12% of the β -globin-mRNA activity were recovered, as measured in the oocyte system. Thus, the insert of Hif-2h hybridises to IF mRNA with about the same efficiency as does β -globin cDNA to β -globin mRNA.

Restriction and sequence analysis of Hif-2h (M. Schwarzstein, N. Mantei and M.S., unpublished results) showed that the insert has 910 base pairs of which 23 are 5'-terminal and 15 are 3'-terminal GC pairs; there is one site each for *Bsp*I (85), *Bgl*II (335) and *Eco*RI (710) endonucleases, two sites for *Pvu*II (125, 425), and three sites for *Ava*II (190, 385, 655) and none for *Hha*I, *Taq*I, *Hind*III, *Hpa*II, *Pst*I and *Bam*HI. (The values in parentheses indicate the distance in base pairs from the *Pst* terminus corresponding to the 5' end of the mRNA.) The orientation of the cDNA insert, as ascertained by nucleotide sequence analysis, was such that the reading direction of the IF cDNA coincided with that of the β -lactamase gene.

Detection of IF activity in *E. coli* strains transformed with Hif-4c-related hybrid plasmids

The isolated Hif-2h *Pst*I fragment was joined to *Pst*I-cleaved pBR322 and to three plasmids, pKT279, pKT280 and pKT287, derived from pBR322 by deletions in the β -lactamase gene; DNA ligated into the *Pst* site of this set can be translated in the three possible reading frames by readthrough from the β -lactamase sequence (K. Talmadge, personal communication). *E. coli* HB101 strains transformed with these hybrid DNAs were *E. coli* HB101 (Z-pBR322(*Pst*)/HcIF-2h-AH1 to -AH4), *E. coli* HB101 (Z-pKT279(*Pst*)/HcIF-2h-AH1 to -AH8) and so forth, or in abbreviated form, 322-AH1 to -AH4, 279-AH1 to -AH8, and so on. S-30 or S-100 extracts, from 24 of the AH and 49 of the SN strains grown to stationary phase, were tested for IF activity. The original Hif-2h-containing strain and many of the AH strains showed IF activity; three of them, 279-AH8, 280-AH3 and 287-AH6, were selected for further testing. Of

Table 2 Characterisation of the insert of hybrid plasmid Hif-4c by the mRNA hybridisation translation assay

DNA fragment	Amount of leukocyte poly (A) RNA (μ g)	Time of hybridisation (h)	IF activity (units ml ⁻¹)
Hif-4c	2.5	16	250; 100
β -globin cDNA	2.5	16	4; 1
Hif-4c	7.5	16	3,000; 1,000
β -globin cDNA	7.5	16	4; 30
Hif-4c	7.5	5	1,000; 1,000
β -globin cDNA	7.5	5	10; 1

The insert of plasmid Hif-4c was excised with *Pst*I, purified by electrophoresis through a 2% agarose gel and recovered by successive adsorption to and elution from hydroxyapatite and DEAE cellulose. 120-ng fragments were linked to each 0.25 cm² DPT paper (B. Seed, personal communication). Pre-hybridisation, hybridisation and elution of RNA were as described elsewhere²². The RNA was injected into oocytes and IF activity was determined after 48 h by the cytopathic effect reduction assay³⁰ (see Table 3 legend).

the 49 SN strains, 16 had IF activity; two of the highest producers, SN35 and SN42, and a negative control, SN32, were further examined. Table 3 shows the results obtained with S-100 extracts of log phase bacteria. IF activities ranged from 100 to 1,000 units per ml of S-100 extract derived from a 20-ml resuspension of the 2.0 g (approximately) of bacterial cells contained in 1 l of culture.

Characterisation of the IF activity produced in transformed *E. coli*

We tested the sensitivity of the IF activity to a protease by incubating S-100 extracts of 287-AH6 and SN35 for 30 min at 37°C with increasing amounts of trypsin. As a control, authentic human Le-IF was mixed with the (inactive) S-100 extract of SN32 (to give a similar protein concentration, 6 mg ml⁻¹) and digested in parallel. In all cases, the activity was partially abolished at 200 μ g ml⁻¹ and completely abolished at 1 mg ml⁻¹ trypsin.

Table 3 IF activity in extracts of transformed *E. coli*

S-100 extracts of <i>E. coli</i> HB101 transformed by:	IF activity (units per ml extract)
a Z-pBR322(<i>Pst</i>)/HcIF-2h	100; 100
b Z-pKT279(<i>Pst</i>)/HcIF-2h-AH8	100; 300
c Z-pKT280(<i>Pst</i>)/HcIF-2h-AH3	1,000; 1,000
d Z-pKT287(<i>Pst</i>)/HcIF-2h-AH6	200; 200
e Z-pBR322(<i>Pst</i>)/HcIF-SN35	1,000; 1,000
f Z-pBR322(<i>Pst</i>)/HcIF-SN42	300; 100
g Z-pBR322(<i>Pst</i>)/HcIF-SN32	0; 0

The IF-cDNA insert of Hif-4c, excised with *Pst*I and purified as described in Table 2 legend, was joined to *Pst*I-cleaved pKT279, pKT280 and pKT287, respectively. *E. coli* HB101 was transformed with these products and tetracycline-resistant colonies were screened by *in situ* hybridisation²⁰ as described by Hanahan and Meselson²⁴, using the Hif-4c *Pst*I fragment nick-translated with [α -³²P]dATP (1,100 Ci mmol⁻¹, NEN) and [α -³²P]dCTP (470 Ci mmol⁻¹, NEN) as labelled substrates³¹. Three clones (b-d) were selected in preliminary assays for IF activity. Clones c-g were from a set of IF-cDNA-containing clones identified among 5,000 *E. coli* HB101 transformed with Le cDNA (preparation B, see Table 1 legend) by *in situ* hybridisation as above. e and f were shown to produce IF in a preliminary screening, and g was chosen as negative control. One-litre cultures of transformed *E. coli* were grown to an A₆₅₀ of about 0.8. The cells (about 2.0 g) were collected, washed with 50 mM Tris-HCl (pH 8), 30 mM NaCl and resuspended in 20 ml of the same buffer. Lysozyme was added to 1 mg ml⁻¹; after 30 min at 0°C, the suspension was frozen and thawed five times and centrifuged at 10,000 r.p.m. for 20 min. The supernatant was centrifuged at 40,000 r.p.m. for 1 h in a Spinco 60 rotor. The S-100 supernatants (about 6 mg ml⁻¹ protein in all cases) were assayed in duplicate by the cytopathic effect reduction assay and their IF content estimated relative to a standard IF preparation. IF activity was determined by the cytopathic effect reduction assay as follows. The IF samples, serially diluted 1:3 were mixed with 10⁵ CCL23 cells in the wells of a microtitre plate (Cooke) in MEM-10% newborn calf serum. After 24 h the medium was replaced by an appropriate dilution of Mengo virus in the same medium. 24 h later the medium was replaced with 0.5% crystal violet, 3% formaldehyde, 30% ethanol and 0.17% NaCl for 15 min; the wells were then washed exhaustively with water.

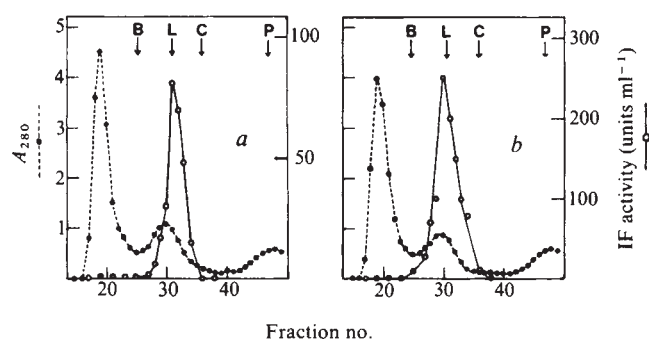


Fig. 2 Chromatography of Le-IF and *E. coli* IF on Sephadex G-100. (a) 0.9 ml S-100 extract of 280-AH3 (500 units, 6 mg protein ml⁻¹) and (b) 0.9 ml of a dilution of human Le-IF (1,000 units of preparation P-IF (ref. 34)) in S-100 extract of SN32 (no IF activity, 6 mg ml⁻¹) were mixed with cytochrome c (0.2 mg) (C), ³²P-phosphate (10⁵ c.p.m.) (P) and ¹²⁵I-labelled β -lactoglobulin (2.5 $\times$ 10⁵ c.p.m.) (L) and chromatographed on a 0.9 $\times$ 49-cm column of Sephadex G-100 in PBS at 4°C. Fractions of 0.7 ml were collected at 2.3 ml h⁻¹. IF activity was measured by the cytopathic effect reduction assay, and radioactivity, A₂₈₀ and A₄₁₀ (cytochrome c) were determined for each fraction. The position of bovine serum albumin (B) was determined in a separate run, relative to C and P.

Table 4 Antibody titres of anti-Le-IF and anti-F-IF measured against different IF preparations

	Le-IF	F-IF	S-100 <i>E. coli</i> extracts	
			SN35	280-AH3
Sheep anti-Le-IF	100,000	3,000	30,000	30,000
Goat anti-F-IF	<10	1,000	<10	<10

About 10 units of each IF preparation were incubated for 1 h at 20°C with different antiserum dilutions and the IF activity was determined by the VSV plaque reduction assay³². The titres are given as the reciprocal of the highest dilution which raises the plaque counts by a factor of 2. The sheep anti-Le-IF was prepared as described elsewhere³³. Human Le-IF was purified to the P-IF stage³⁴.

Human Le-IF is stable at pH 2 (ref. 21). S-100 extracts of 280-AH3 and SN35, as well as 250 units human Le-IF mixed with (inactive) S-100 extract of SN32, were dialysed overnight against 0.1 M NaCl and 50 mM glycine-HCl (pH 2) buffer and then 5 h against phosphate-buffered saline (PBS). A precipitate was removed by centrifugation and the supernatant assayed by both cytopathic effect reduction and plaque reduction. In all cases the initial IF activity was recovered in full.

To compare the MWs of authentic Le-IF and the IF activity in transformed *E. coli* (*E. coli* IF), S-100 extracts were chromatographed on Sephadex G-100 columns. IF activity moved with a K_{av} of 0.46 in the case of 280-AH3 (Fig. 2a), 281-AH6 and SN35 (data not shown), which was slightly slower than authentic Le-IF (mixed with S-100 extract of SN32, Fig. 2b); the difference may, however, not be significant.

We compared the serological properties of authentic IFs and *E. coli* IFs. As shown in Table 4, sheep anti-human Le-IF had a similar titre against Le-IF and *E. coli* IF of SN35 and 280-AH3, and was $\frac{1}{30}$ th as active on fibroblast IF (F-IF); goat anti-human F-IF was active only against F-IF. Thus, *E. coli* IF is immunologically similar to Le-IF, and quite distinct from F-IF.

Both authentic Le-IF and *E. coli* IFs show specificity in regard to the cells on which they will act: they are most active on human cells, less active on monkey and mouse cells and inactive on chick cells (Table 5). It is not clear whether the relatively high activity of *E. coli* IF on monkey cells is significant; further experiments with the purified material are necessary.

As shown by Kerr^{4,5} and others, treatment of cells with IF increases 10- to 15-fold their level of oligoisoadenylate synthetase, an enzyme that condenses ATP to ppp(A2'p)_n5'A ($n = 1-4$). Cells were treated with various IF preparations and the cell extracts assayed by measuring the ³H radioactivity transferred from ³H-ATP to the dephosphorylation products of pppA2'p5'A and ppp(A2'p)₂5'A, namely (2'-5')ApA and (2'-5')ApApA. As shown in Table 6, (2'-5')ApA radioactivity was six- to ninefold higher in cells treated with Le-IF or *E. coli* IF than in controls treated with an inactive *E. coli* extract, and (2'-5') ApApA radioactivity was more than 14-33 times higher than in controls; there was no significant difference between the activity of Le-IF and S-100 extracts of 280-AH3 and SN35.

Table 5 IF activities measured on different cell types

Cells Expt 1	Le-IF	Interferon F-IF	Interferon activity <i>E. coli</i> S-100 extracts	
			280-AH3	SN35
Human U amnion	6,000	2,000	600	600
Monkey Vero	600	600	350	350
Monkey GMK	350	200	350	110
Primary chick embryo fibroblasts	<20	<20	<20	<20
Expt 2	Le-IF	mouse-IF	<i>E. coli</i> S-100 extracts	
			287-AH6	SN35
Human CCL23	1,000	ND	300	1,000
Mouse L929	40	120	40	120

Human Le-IF was preparation P-IF (ref. 34), mouse IF was the NIH standard. U cells were maintained by K.C. All cells were challenged with VSV, except for CCL23 cells, where Mengo virus was used. Experiment 1 was assayed by plaque reduction, experiment 2 by the cytopathic effect reduction assay. ND, Not done.

Thus, by all criteria tested, *E. coli* IF is very similar to authentic Le-IF, although, of course, the molecular structure may well differ in various respects.

Discussion

A strain of *E. coli* containing IF-cDNA was identified by an IF-mRNA hybridisation translation assay in which DNA from successively smaller pools of strains was screened. Because only 4 of 12 groups of 512 clones had originally given a positive response, we were surprised to find 5 IF clones in a selected group of 64. It is probable that, when used on large pools, the assay was at borderline sensitivity and only detected groups and subgroups particularly rich in IF-cDNA clones. The subsequent screening of 5,000 colonies using an IF-cDNA probe revealed 185 positive clones, a frequency of about 1:27. Taking into account the fact that the poly(A) RNA used to generate the clones had been enriched about 10-fold with respect to IF mRNA, the proportion of IF mRNA in poly(A) RNA from induced leukocytes was not less than 1:270.

The identification of the 910-base pair insert in Hif-2h as a cDNA copy of human Le-IF rests on two lines of evidence: (1) its capacity to hybridise selectively to IF mRNA, and (2) its ability to direct the synthesis, in *E. coli*, of a polypeptide with the

Table 6 Levels of oligoisoadenylate synthetase in human cells treated with Le-IF or *E. coli* IF

Cells treated with:	Cell protein (μg)	³ H-A in oligoisoadenylate (% of recovered radioactivity)	
		ApA	ApApA
1. S-100 extract of SN35 (200 units IF ml ⁻¹)	7.6	1.4	<0.1
	38	5.2	1.4
2. S-100 extract of 280-AH3 (200 units IF ml ⁻¹)	7.6	1.5	0.1
	38	7.8	3.3
3. S-100 extract of SN32 (no IF)	7.6	<0.1	<0.1
	38	0.9	<0.1
4. Le-IF (P-IF) (200 units IF ml ⁻¹)	7.6	1.3	0.25
	38	8.0	2.1

Confluent CCL23 cell monolayers in 50-mm dishes were treated with a mixture of 1 ml *E. coli* S-100 extract and 4 ml minimal essential medium (MEM)-10% newborn calf serum or a dilution of Le-IF (P-IF) in 5 ml medium. After 20 h the cells were lysed and supernatants prepared as described elsewhere³⁵. Varying amounts of lysate were adsorbed to poly(rI).(rC)-Sephacrose and incubated as described elsewhere³⁵, except that ³H-ATP (specific activity 40 Ci mmol⁻¹) was used instead of ³²P-ATP. After treatment with bacterial alkaline phosphatase, the products were separated by electrophoresis using ApA and ApApA as markers³⁵. The paper was cut into strips and the radioactivity determined by scintillation counting. Most radioactivity was recovered in adenosine. 100% radioactivity was 3-5 × 10⁴ c.p.m.

biological activity of IF. The polypeptide has properties of human Le-IF in that it induces a virus-resistant state in human cells, to a lesser extent in monkey and mouse cells and not in chick cells, and is neutralised by antibody to human Le-IF but not to human F-IF. Moreover, *E. coli* IF stimulates the activity of isoadenylate synthetase in human cells to the same extent as does authentic Le-IF.

The IF-cDNA plasmids were constructed to allow synthesis of an IF molecule fused to part of β-lactamase. It seems likely, however, that the biologically active material is a non-fused polypeptide, because its formation is directed by hybrids derived from each of the three pKT plasmids and is therefore independent of the reading frame resulting from the construction. Moreover, a fused β-lactamase fragment should contribute 180 amino acids when the IF cDNA is inserted in the *Pst*I site of pBR322, but not more than 26 or 29 amino acids when it is linked to pKT280 or pKT287 (K. Talmadge, personal communication); in fact, there is no detectable difference in the size of the biologically active IF polypeptides made by the three strains. At the structural level, *E. coli* IF probably differs from authentic Le-IF by the absence of appropriate glycosylation. Also, it is possible that *E. coli* IF consists of the Le-IF sequence preceded by a signal sequence, as nucleotide sequence analysis of the cloned IF cDNA revealed a region coding for 22 amino

acids which follows the first AUG and precedes the stretch coding for mature IF (M. Schwarzstein, N. Mantei and M.S., unpublished results).

We do not know whether *E. coli* IF has the same specific activity as authentic Le-IF. If this were the case, the amount of active IF produced in transformed *E. coli*, about 20,000 units per l of culture, would correspond to one to two fully active molecules per cell. This would be consistent with the occurrence of rare translational events at the physiological initiation site of the IF sequence, and appropriate modifications of the hybrid plasmid should allow a considerable increase in the yield of active IF. If, however, lack of appropriate glycosylation diminishes the activity of the molecule, we shall have a problem on our hands.

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Note added in proof: Taniguchi and his colleagues have recently identified by nucleotide sequence analysis (T. Taniguchi, personal communication) a fibroblast IF cDNA hybrid prepared from induced fibroblast poly(A) RNA and selected by hybridisation procedures (T. Taniguchi *et al.*, *Proc. Jap. Acad.* **55**, Ser. B, 464–469 (1979)).

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Lethal metabolism of precocene-I to a reactive epoxide by locust corpora allata

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The corpora allata from adult female Locusta migratoria rapidly metabolise precocene-I to a geometric mixture of dihydrodiols, and simultaneously suffer extensive macromolecular labelling. This could explain the selective cell death which precocenes induce, and focuses attention on the high level of epoxidase in these insect organs.

It is not known how the natural product precocene-I (ref. 1), its congener precocene-II (ref. 1), or related analogues², cause selective necrosis^{3,4} of cells in the corpora allata (CA) of sensitive insects. Therefore, it has not been possible to rationalise the activity or inactivity of precocene analogues², or to explain the marked species specificity of these compounds. An early suggestion⁵ that precocenes might act on the brain, which normally controls the CA, has received no further experimental support. Initial experiments on the isolated CA of a relatively insensitive species were inconclusive⁶. More positive evidence for a direct action of precocenes on the CA comes from studies on CA of adult female *Oncopeltus*⁷, in which 87% of CA failed to elicit a positive response in a transplantation bioassay after culture for 5 days in a medium containing 4.5×10^{-5} M precocene-II. The likelihood of an oxidative biotransformation being crucial to the action of precocene was raised by our recent finding⁸, that its action on both *Oncopeltus* and *Locusta* is suppressed by co-treatment with certain benzodioxole mixed function oxidase inhibitors, although these seem to protect precocene against metabolic inactivation. We speculated⁸ that a crucial activating metabolism of precocenes may occur within the CA, which might explain their tissue specificity. Accordingly, we have synthesised radiolabelled precocene-I, and

studied its metabolism by CA and non-target tissues of adult female *Locusta migratoria*, *in vitro*. Our results strongly indicate that the CA rapidly oxidise precocene-I at the 3,4 double bond to a highly reactive⁹ epoxide, much of which forms covalent attachment to cellular macromolecules. We suggest that this may be the basis of the cytotoxic action of precocenes. By contrast, non-target tissues are not subjected to intense labelling of macromolecules, which may be important in determining the selective action of precocenes. Our hypothesis focuses attention on the enzymic competence of the CA to oxidise precocene-like molecules to highly reactive epoxides.

Precocene metabolism and covalent attachment to macromolecules

We synthesised [4-³H]precocene-I (2,2-dimethyl-7-methoxychromene) at 34 mCi mmol¹⁰. Authentic reference compounds I–IV (Fig. 1) were prepared for identification of possible metabolites by published methods^{1,9,11}, all being known compounds. 2,2-Dimethyl-7-hydroxy-chromene¹² was prepared by the method of Bowers *et al.*¹ from the corresponding chromanone¹¹. The dihydrodiols (IV)¹³ were prepared from the *m*-chloroperoxybenzoic acid adducts to precocene-I (ref. 9) after separation of *cis* and *trans* forms (Waters Prep system 500,

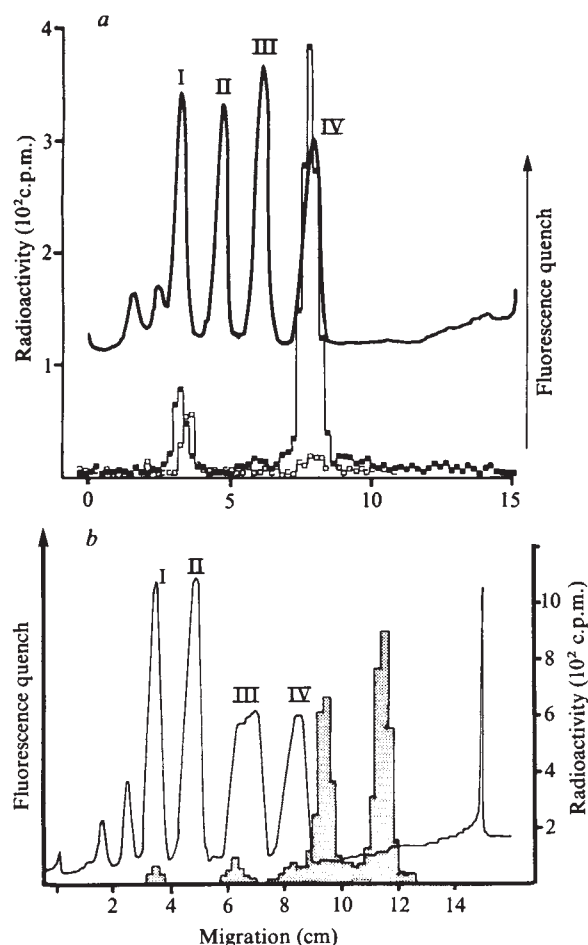


Fig. 1 Radio-TLC analysis of precocene-I metabolites produced by several locust tissues *in vitro*. *a*, Five lots of two pairs of CA from 14-day-old adult female *Locusta migratoria*, reared as described elsewhere⁸, were incubated by shaking in the dark at 30 °C in 0.1 ml each of TC 199 medium (2 mg ml⁻¹ FicollTM, 20 mM HEPES buffer, pH 7.2 at 30 °C) with [4-³H]precocene-I (34 mCi mmol⁻¹; 99.3% radiochemical purity by quantitative RP-TLC) at an initial concentration of 0.101 mM, for 3 h. Incubation tubes and glass transfer pipettes were coated with 5% aqueous Carbowax 20 M. The incubation was stopped by exhaustive extraction on the centrifuge with ethyl acetate (containing 20–50 µg nonradioactive reference compounds) the extract dried with anhydrous sodium sulphate, and the volume reduced under nitrogen flow before TLC application. RP-TLC was carried out on Merck 0.25 mm KC-18 indicator plates, pre-washed in alumina purified ethyl acetate then AR methanol, after application to 1.5 × 1.5-cm area. The samples were pre-focused twice with redistilled methanol then developed, in a darkened sandwich chamber for 15 cm in methanol:water (7:3). Authentic internal reference compounds were localised by fluorescence quenching densitometry (continuous line) (Vitatron TLD), and radioactivity localised by windowless gas flow counting (Berthold scanner, 1-mm window, scaler mode, background 3 c.p.m.). Reference compounds: I, 2,2-dimethyl-7-methoxy-chromene (precocene-I); II, 2,2-dimethyl-7-methoxy-chroman-4-one; III, 2,2-dimethyl-7-hydroxy-chromene and 2,2-dimethyl-7-methoxy-chroman-4-ol (poorly resolved); IV, *cis*, *trans* 3,4-dihydroxy-2,2-dimethyl-7-methoxy-chromene (precocene-I dihydrodiols). ■, CA incubation; □, control incubation without tissue. *b*, Similar experiment to *a*, using Malpighian tubules, except that the initial precocene concentration was 0.14 mM, and the combined incubations were blended in 5 ml of redistilled ethyl acetate before repeated extraction. Qualitatively similar results were obtained with ligatured mid-guts and abdominal fat body sheet.

two series mounted silica PrepPAKs 5.7 × 30 cm, 30% diethyl ether in pentane) by base hydrolysis (1M NaOH, 50% aqueous ethanol) 30 min at room temperature). All compounds were characterised by appropriate physical methods. When CA from reproductively mature female *Locusta* were incubated in tissue culture medium containing labelled precocene-I for 1–5 h, and the incubations partitioned between either salt solution/chloroform + methanol, or /ethyl acetate, negligible radioactivity (<1.5%) was found in the aqueous phase. Analysis of the chloroform layer by reversed-phase thin layer chromatography (RP-TLC) suggested that there was only one important metabolite (96% of products) in the CA, which co-chromato-

graphed with a mixture of *cis*/*trans* 3,4 dihydrodiols, whereas the selected non-target tissues (abdominal fat body, ligatured mid-gut and Malpighian tubules) produced varying quantities of material of intermediate polarity (Fig. 1). In two experiments, half the chloroform layer was also analysed by normal phase TLC (silica gel; ethyl acetate: xylene, 25:75) with mutually consistent results (data not shown). When the CA were separated from the incubation medium at the end of the experiment by gland transfer¹⁴, and extracted separately, more than 99% of the dihydrodiol present was found to be in the surrounding medium. Analysis of the extracted CA revealed up to 400 pmol precocene equivalents per gland pair bound to non-extractable residues, whereas the radioactivity associated with non-extractable residues from the non-target tissues was much less (~4–15 pmol per animal equivalent) after 3 h incubation. In three experiments, duplicate CA samples were subjected to further extraction with 10% TCA before solubilisation and radioactivity determination, with only slight (1–8%) loss of radioactivity. We take this to be evidence of covalent attachment, but have yet to demonstrate either its nature or cellular location. Depending on the initial concentration of precocene in these experiments (range 1.7×10^{-5} – 4.4×10^{-4} M) CA macromolecules were labelled to the extent of 7.8–80 nmol per mg tissue protein. These are very high values. For example, the lowest value is 80 times the degree of labelling of rat liver protein *in vivo* following administration of an hepatotoxic dose of bromobenzene, even when protection by endogenous glutathione is at its minimum¹⁵.

We studied the time course of precocene–dihydrodiol formation and m.cromolecule labelling in the CA by replacing the substrate medium every 30 min (Fig. 2a), thereby maintaining the precocene concentration within a defined range. Figure 2a shows a trend towards progressively increased rate of dihydrodiol release over a period of 2.5 h. Macromolecules are labelled at a constant rate in these conditions, after an extrapolated lag period of 9 min. We also examined the time course of metabolism during incubations in which an initially high precocene concentration was allowed to decline exponentially for 3 h. Figure 2b shows one such experiment, resulting in a final concentration of 7×10^{-5} M, and it is clear that dihydrodiol formation and macromolecule labelling proceed unabated over this period. Other experiments showed that the metabolic rate varies with, but is not proportional to, the precocene concentration over the range studied.

Stereochemistry of precocene dihydrodiols

To identify the dihydrodiols further, we isolated 2.9×10^5 d.p.m. by RP-TLC, and analysed aliquots by high resolution silica HPLC (Fig. 3). Of the radioactivity recovered (94% of that applied), 96% co-chromatographed exactly with authentic internal markers of *cis* and *trans* dihydrodiols, with radioactivity in the ratio *trans*:*cis*, 62:38. We also examined the very much smaller quantities of dihydrodiol produced by the three other tissues studied, where it does not accumulate progressively, and obtained *trans*:*cis* ratios of 63:37 to 80:20.

We believe that the high *cis* content of the dihydrodiol produced by the CA is important evidence of trigonal hybridisation in a reaction intermediate, namely the 3-OH, 4-carbocation which would result from proton catalysed opening of the 3,4 epoxide ring; it is known that chemical hydration of precocene-I 3,4-epoxide yields significant quantities (21–36%) of *cis* dihydrodiol in a wide variety of conditions (ref. 9 and A. F. H., A. P. Ottridge, G. E. P., R. C. J. and K. M. Stott, unpublished results). This proposed hydration mechanism, and the high reactivity of precocene-I epoxide⁹, compares readily with that deduced for the reactive epoxides of benzo(α)pyrene 7,8-dihydrodiol¹⁶ and many other mammalian carcinogens¹⁷. This contrasts markedly with the relatively stable epoxide group in the natural product (juvenile hormone) of the CA, whose acid-catalysed hydration is predominantly SN2 in character and leads to 97% *trans* dihydrodiol¹⁸. Enzymic hydrolysis of epoxides typically yields *trans* dihydrodiols¹⁹. Apparently, dihydrodiols

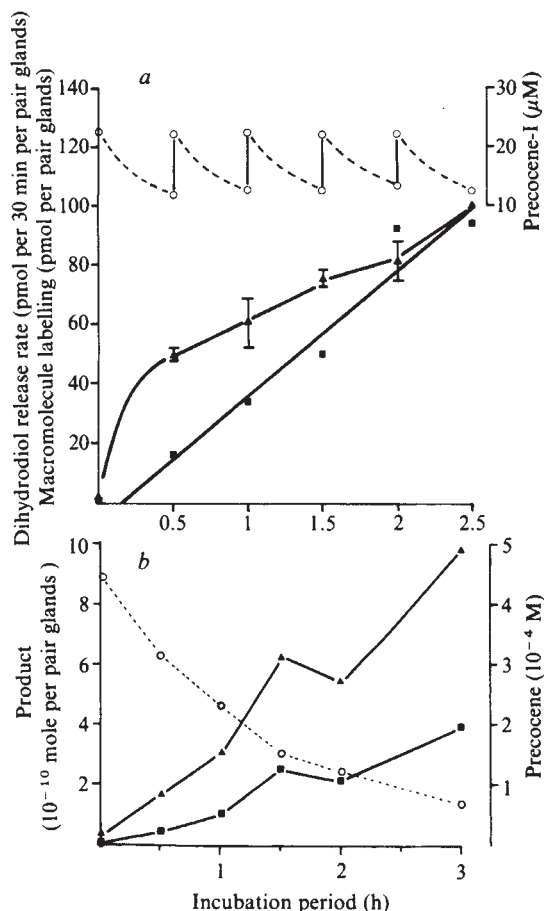


Fig. 2 Progressive formation of dihydrodiols and labelled macromolecules from [4-³H]precocene-I by *Locusta* CA, *in vitro*. Glands were incubated as in Fig. 1, but at different precocene-I concentrations. Δ , Incubations were extracted with 25 volumes of CHCl₃:methanol (10:1) and the extracts analysed by RP-TLC for precocene-I dihydrodiol content. $\blacksquare$, Labelled macromolecules were estimated after removal of the CA from the aqueous phase on stainless steel loops and extraction twice in 0.5 ml methanol (6 h), before solubilisation (0.2 ml Lumasolve, LKB, 2 h, 45 °C with shaking) and liquid scintillation counting (4.7 ml Lumagel, LKB, neutralised with glacial acetic acid, shaken for 1 h) in a Wallac 81000 calibrated on AES ratio with standard tritiated water (Amersham), in minivials. Radioactivity on RP-TLC plates was qualitatively localised by gas-flow β -imaging (Panax Betagraph) and correlated with the fluorescence quenching profile of authentic *cis/trans* precocene-I dihydrodiols, which were recovered by scraping into micro-filter tubes under suction, and quantitatively eluted with 8 $\times$ 0.2 ml methanol into 9.5 ml of toluene-based scintillator²⁵ for scintillation counting. *a*, CA were bilaterally randomised between five incubation tubes (four glands per 0.1 ml culture medium) and incubated for different total periods, in duplicate, in 2.3×10^{-5} M [4-³H]precocene-I. The medium was recovered by aspiration every 30 min, for analysis by RP-TLC, and replaced with fresh medium, until the end of the experiment. The degree of cumulative labelling of cellular macromolecules was determined at the end of each incubation; each point represents the mean of two separate observations, and the straight line is a fitted linear regression ($r = 0.96$). The stock solution of substrate in TC 199 was stable at 0 °C. $\circ$, The concentration of precocene-I at the end of each 30-min interval was determined by subtracting the radioactivity found in the dihydrodiol zone after RP-TLC, from the total radioactivity in the medium (determined by scintillation counting of a 5- μ l aliquot of a 1:1 mixture and ice-cold methanol prepared immediately after aspiration of the medium from the CA). The points are variously the means of 2–10 estimations, and standard errors are indicated where appropriate. The saw-tooth line joining the concentration points is purely illustrative (compare with *b*). The final balance of radioactivity was completed by elution of the plastic incubation caps in toluene (10% ethanol) scintillant. *b*, Similar to *a*, except that the incubations were initiated at 4.45×10^{-4} M [4-³H]precocene-I, and the media were not replaced during the course of the incubations. Each point is the mean of two determinations.

are important metabolites of precocene-II in whole insects of several species²⁰, and in an isolated non-target tissue (fat body) from two insensitive species²¹, but their stereochemistry has not been reported. We have used standard methods²² to investigate epoxide hydrolase activity in isolated *Locusta* CA with [12-³H]C₁₆ juvenile hormone (340 mCi mmol⁻¹) as substrate, and

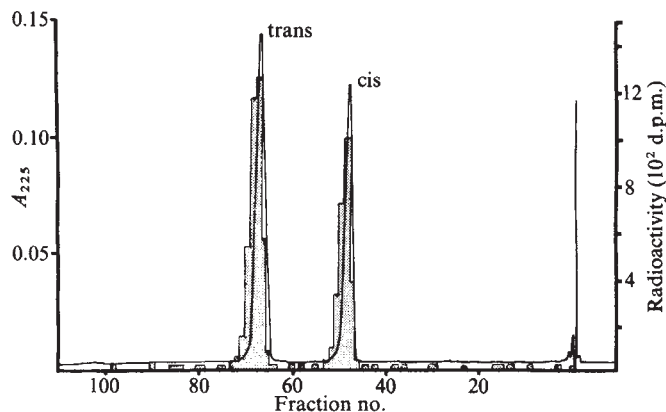


Fig. 3 High resolution liquid chromatograph of precocene-I dihydrodiols produced by *Locusta* CA *in vitro*, and isolated by RP-TLC. Radioactivity in the dihydrodiol zone after RP-TLC (Fig. 1a) was eluted with a total of 1.5 ml redistilled dichloromethane, filtered and evaporated. The sample was dissolved in HPLC solvent, and 4.8 μ l (6,914 d.p.m.) analysed on 5 μ m Zorbax-sil (250 $\times$ 4.6 mm; diethyl ether:pentane:2-propanol (49.9:49.9:0.2), 1.5 ml min⁻¹). Fractions of effluent were collected every 12 s for determination of radioactivity.

failed to find any (detection limit ~ 1 pmol h⁻¹ per gland pair), which is in accord with the physiological function of these glands. Thus, if dihydrodiol formation in the CA is a consequence of epoxidation, the final hydration step is almost certainly a spontaneous chemical event, proceeding via a reactive carbocation which would be expected to alkylate cellular nucleophiles^{9,15} in competition with its reaction with water. Apparently, our proposed pathway may account for both the stereochemistry of the dihydrodiols and the labelling of cellular macromolecules.

Sensitivity to a mixed function oxidase inhibitor

We sought evidence for an initial lethal synthesis involving epoxidase action on precocene-I by studying the effect of a mixed function oxidase (MFO) inhibitor on precocene metabolism in the CA. Many MFO inhibitors are known to inhibit juvenile hormone (JH) biosynthesis in both whole cockroach CA²³ and homogenates thereof²⁴. As part of a larger study

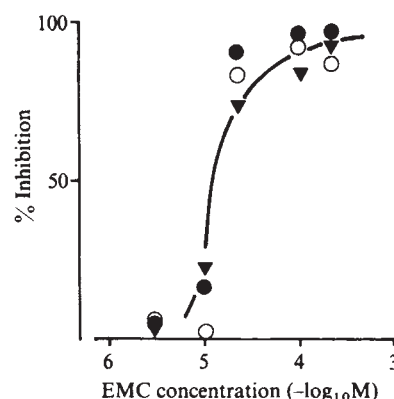


Fig. 4 Inhibition of precocene-I and farnesenic acid metabolism in *Locusta* CA by EMPB, *in vitro*. CA were bilaterally randomised between control and experimental tubes, and pre-incubated with the appropriate concentration of EMPB for 30 min before addition of radiolabelled substrates²³. When precocene-I was the substrate it was present at an initial concentration of 0.12 mM, and radiolabelled incubation was for 2 h. Precocene-I ($\bullet$) dihydrodiols and labelled macromolecules ($\circ$) were determined as described in Fig. 2 legend. When farnesenic acid was the substrate the media contained [12-³H]2E,6E-farnesenic acid (309 mCi mmol⁻¹; 2.6×10^{-5} M), and [Me-¹⁴C]methionine (Amersham; final activity 35.4 mCi mmol⁻¹; 1.6×10^{-4} M). Methyl farnesoate and juvenile hormone ($\blacktriangledown$) were quantified by extraction, silica gel TLC and liquid scintillation counting²⁵. Inhibition values were the same whether calculated on ³H or ¹⁴C radioactivity. The data are composited from two separate experiments, each point representing a single observation except those at 10^{-4} M EMPB, which are duplicates.

(G.T.B., G.E.P. & J. A. Cocks, unpublished) we synthesised ethyl (2E)-4-(3,4-methylenedioxyphenoxy) butanoate (EMPB) by transesterification (BF₃/ethanol) of the corresponding methyl ester derived from the esterification of sesamol with methyl 4-bromo-2E-butenate (K₂CO₃/dimethoxyethane, 60 °C). The compound was 98% pure by HPLC (5 µm Zorbax-sil, N_{eff} = 9,300, pentane: diethyl ether: s-propanol 9.9: 89.9: 0.2). We tested this compound in a standard assay for inhibition of spontaneous C₁₆JH biosynthesis in cockroach CA²³, and found an IC₅₀ of 8×10^{-6} M coupled with a large accumulation of methyl farnesoate, unlike some other MFO inhibitors which seemed to inhibit both epoxidation and esterification reactions in the biosynthetic pathway²³. EMPB also inhibits C₁₆JH biosynthesis from exogenous farnesic acid²⁵ in *Locusta* CA (Fig. 4), although it seems to be slightly less active in this system. More importantly, Fig. 4 shows that adult female *Locusta* CA are almost equally sensitive to EMPB in respect of: (1) epoxidation of internally generated methyl farnesoate; (2) precocene-I dihydrodiol formation; and (3) precocene labelling of macromolecules. We observed no significant diversion of precocene-I metabolism towards other products in the presence of EMPB. These results demonstrate that an oxidative enzyme is crucial to both ultimate pathways of precocene metabolism in the CA. Kinetic studies on a partially purified methyl farnesoate epoxidase from adult female *Locusta* CA have revealed high

levels of activity, with typical $\nu_{\max}$ values of 1–2 nmol min⁻¹ per mg total tissue protein (R. Feyereisen & G. E. P., unpublished results). We have yet to demonstrate that precocene-I is a lethal alternative substrate for this enzyme, but suspect this to be the case, because the specialised endocrine function of the glands makes it unlikely that they will be rich in other oxidases involved in general detoxifying metabolism.

Conclusions

The oxidative activation of precocene-I in *Locusta* CA may be compared with the various types of oxidation which are believed to be crucial to the action of aromatic cytotoxins¹⁵, necrogenic neurotransmitter analogues²⁶ and carcinogenic polycyclic aromatic hydrocarbons^{16,17} in vertebrate tissues. In these cases, the mechanisms of possible importance include the generation of epoxides^{15–17}, quinonoids^{26,27} and reactive forms of oxygen^{28,29}. Causative interpretation of these findings has been particularly difficult in the case of polycyclic aromatic hydrocarbons because of the multiplicity of metabolic pathways²⁹. In contrast, the present studies suggest that precocenes are metabolised by a single enzymic pathway in the CA; which should facilitate investigation of their selective cytotoxic action.

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LETTERS

Emission at 3.3 µm and evidence for dust in 3C273

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The presence of dust grains in a variety of astronomical environments has been established from many IR observations¹. Of particular interest is whether dust exists in the highly energetic quasars, and in the less energetic (but apparently similar) Seyfert 1 galaxies. Whilst both quasars and Seyfert 1 galaxies exhibit enhanced radiation over just the wavebands expected of thermal dust emission, the interpretation of this enhancement is a matter of controversy². The origin of the non-thermal radiation that quasars emit at other wavebands is not understood; hence a non-thermal origin of their enhanced IR continua cannot be excluded. The existence of dust also has relevance to current discussions on the hydrogen emission-line ratios, for the weakness of the broad Ly α emission can possibly be explained by absorption on dust grains^{3,4}. I present here circumstantial evidence that dust exists in the quasar 3C273, by demonstrating the existence of the 3.3 µm line in emission.

Emission at 3.3 µm has been found in many galactic and extragalactic objects. In the most complete reviews of this feature^{5,6}, the wavelength was given as 3.28 µm, and a qualitative correlation with the presence of dust grains was noted. A gas-phase origin of the emission was shown to be unlikely because of the intrinsic width of the feature⁵. Recently an identification with C–H bonds (for example, methane) on the surface of dust grains has been proposed⁷.

Spectroscopic observations were made of the quasar 3C273 using the IR photometer-spectrometer on the 3.9 m Anglo-Australian Telescope. In its spectroscopic mode this instrument makes use of a circular variable filter and is a single-channel fast-scanning spectrometer. A total of 1,800 scans through the spectrum were made in a period of 2.5 hours; the spectral region covered was 3.68–3.96 µm. The spectral resolution was 0.08 µm, and samples were taken every 0.02 µm. For the redshift of 3C273 the expected wavelength of the 3.28 µm feature is 3.80 µm.

The data are presented in Fig. 1. An unresolved emission feature is clearly seen rising about 40% above the continuum and having an equivalent width of 170 ± 40 Å. The rest wavelength of this feature is 3.26 µm. This differs from the observed wavelength of the feature in other objects by 0.3 of the spectral resolution, an amount roughly equal to the uncertainty of the wavelength calibration in this spectral region.

The presence of the 3.28-µm feature is strong circumstantial evidence for the existence of dust somewhere in the immediate

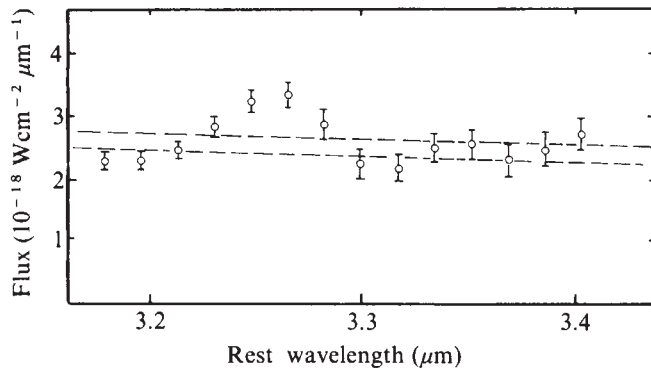


Fig. 1 Spectrum of 3C273 in the rest wavelength range 3.2–3.4 μm . The two broken lines indicate the 1- σ bounds to the estimated continuum based on independent broad-band IR measurements made on the same night (9 April 1979).

vicinity of 3C273. This in turn makes more credible the association of the IR enhancement with thermal radiation from dust. Whether that dust lies within the emitting gas, and therefore can absorb a significant fraction of the Ly α emission, cannot be determined from present data. If, however, the 1–10 μm continuum⁸ in 3C273 is interpreted as thermal radiation from

that dust, the black-body colour temperatures implied range from a few hundred to about 1,000 K. Such temperatures would be found at distances of the order of 10 to 100 pc from the central energy source for a luminosity of about $10^{13} L_{\odot}$ (ref. 8). The dust therefore lies outside at least part of the nuclear region wherein the broad emission lines probably originate. In a study of the effects of dust on the emission-line spectra of quasars, Ferland and Netzer⁹ concluded that the weakness of Ly α could possibly be explained better by dust outside the emission region than within it. However, such statements are inevitably provisional as there is no guarantee that the dust is distributed with spherical symmetry about a quasar's nucleus.

It would be of interest, although difficult, to search for the emission features at 7.8 and 8.7 μm , which are present in most galactic sources having 3.3- μm emission⁶, and which are redshifted to 9.0 and 10.1 μm , both convenient parts of the atmospheric window.

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A probable 1970 hard X-ray outburst by 4U0041+32

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During a balloon test flight on 4 February 1970, an engineering model of the UCSD Cosmic X-ray Experiment, later flown on the OSO 7 satellite, observed what was apparently an unknown high-latitude X-ray source at $\alpha = 0^{\text{h}} 45^{\text{m}} \pm 5^{\text{m}}$, $\delta = 33^{\circ} \pm 3^{\circ}$. The source had a 25–300 keV flux of about 6×10^{-2} photons $\text{cm}^{-2} \text{s}^{-1}$, and a very hard spectrum over that energy range. By the time of a subsequent balloon flight on 25 September 1970, the intensity had faded to below 10^{-2} photons $\text{cm}^{-2} \text{s}^{-1}$ (99% confidence). Recent observations by several experimenters have led us to associate the balloon observation with the highly variable source 4U0041+32. Certain characteristics of this source, such as its high galactic latitude and hard spectrum extending to ~ 300 keV, set it apart from other known transients.

At the time of the 1970 balloon flights there was no known source in the region. The 2U Catalogue¹ of 1972 contained an entry, 2U0043+32, compatible with the balloon position, but the source was weak (7.8 ± 0.6 Uhuru counts) and not described as variable. Variability was first suggested by MIT OSO 7 data², and confirmed by Murray and Ulmer³ in 1976, based on further analyses of Uhuru data. In 1977 February an X-ray outburst⁴ occurred, during which the source reached an intensity of ~ 50

Uhuru counts. SAS 3 observations⁵ over the following several months improved the source position to a 1 arc min radius error circle, allowing a tentative optical identification by Charles *et al.*⁶. The identification was based on weak He II $\lambda 4686$ emission seen on one of several nights' spectrophotometric observations of the $V \sim 19.2$ mag G-type candidate star. The faintness of the candidate (and the absence of bright stars nearby) seems to require a distance of at least 5 kpc, placing the high latitude object within the galactic halo. Evidence for a possible 11.6-day periodicity has been reported by Watson and Ricketts⁷ from Ariel 5 data, while the SAS 3 observations⁵ limit short-period pulsations to $< 7\%$ of the total flux. The aforementioned discoveries, together with others establishing the hard X-ray transients as fairly common phenomena^{8–12}, have now made it clear

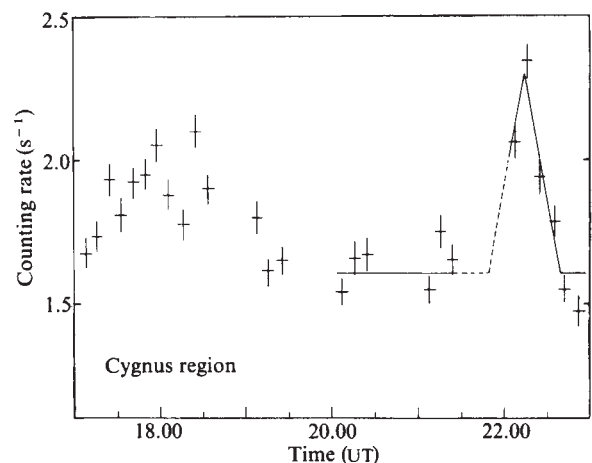


Fig. 1 20–50 keV counting rate versus time during the February 1970 balloon flight. The solid line shows the expected collimator response to a point X-ray source at $\alpha = 0^{\text{h}} 45^{\text{m}}$, $\delta = +33^{\circ}$. The high rates early in the flight are due primarily to Cygnus X-1. Gaps in the data indicate times when the instrument was being calibrated or when the blocking crystal was in the detector aperture.

that the 1970 balloon observation was probably of a prediscovery outburst of 4U0041+32.

The observation was made by a scintillation telescope¹³ consisting of a 64 cm² area, 1-cm thick NaI(Tl) central detector collimated to a $\sim 7^\circ$ FWHM field-of-view by a surrounding CsI(Na) shield in active anticoincidence. As the flight was primarily for test purposes, the instrument was mounted vertically beneath a rotating table that contained an opening, a calibration source, and a NaI blocking crystal. To obtain the maximum amount of diagnostic information, individual central detector events in the 15–550 keV range were pulse-height analysed in a 256-channel linear PHA and telemetered to the ground along with associated shield coincidence tags. The actual anticoincidence vetoing was carried out during subsequent computer analysis. In addition, nearly all central detector and shield discriminator rates and many other housekeeping functions were continuously monitored. Background spectra and calibrations were taken hourly utilising the rotating table, except during the early part of the flight when the Cygnus region crossed the detector aperture.

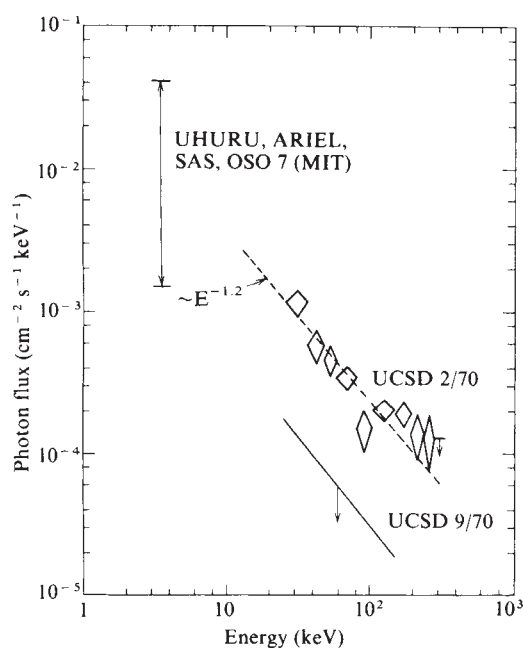


Fig. 2 Spectral Information on 4U0041+32. The range of intensities seen in the ~ 2 –10 keV range is shown together with the two 1970 balloon observations.

Figure 1 shows 20–50 keV counting rates observed during the flight. The low rates reflect post-flight processing to include the effects of the anticoincidence shielding. A source transit apparently occurred shortly after 22.00 UT. Although the data were never published (due, among other reasons, to the second flight's failure to confirm the source), the apparent transit has all the characteristics expected for a real cosmic X-ray source. First, the hard spectrum of the excess counts (Fig. 2) rules out several alternative possibilities, such as an increase in detector (PMT) noise, an X-ray aurora, or most precipitating particle events. Second, there was no significant increase or decrease in any of the instrument discriminator counting rates during the transit. Possibilities such as a change in anticoincidence efficiency due to degraded shield performance, or any increase in the overall radiation environment, are, therefore, largely excluded. Furthermore, the radiation causing the excess counting rate must have formed a well-collimated beam, because the shield rates, which are sensitive to diffuse or isotropic radiation but relatively insensitive to unidirectional radiation beams, were also constant within the statistical uncertainties. As it is highly

implausible that charged particles could form such a beam and maintain it stably over the ~ 30 min duration and ~ 50 km spatial extent (the balloon drift rate was ~ 100 km h⁻¹) of the observation, the radiation beam must have been a beam of photons—an X-ray source. Finally, the time history of the excess counting rate (Fig. 1) agrees well with that expected from the collimator response to a point source at $\alpha = 0^{\text{h}} 45^{\text{m}} \pm 5^{\text{m}}$, $\delta = 33^\circ \pm 3^\circ$. If we use the scatter in the eight background data points between 20.00–21.30 UT and 22.30–23.00 UT to determine the errors (thereby obtaining errors about 30% larger than those expected from Poisson statistics), the 20–50 keV source transit shown in Fig. 1 has a significance of $\sim 10\sigma$.

Given the moderate sensitivity of our balloon experiment, it seems unlikely that we have obtained the only observation of a high latitude ($b \sim -30^\circ$) variable or transient X-ray source located within 3° of the known variable 4U0041+32. Unfortunately, our incomplete knowledge of the occurrence of this type of object, does not allow us to place a reliable quantitative value on the probability of such a coincidence. Note also that the weak (3 Uhuru counts s⁻¹) source 4U0041+36 could conceivably have been responsible for the 1970 outburst. Nonetheless, as it seems most likely that the balloon source and 4U0041+32 are the same, we shall base the following discussion on that assumption.

The spectrum that we obtained for 4U0041+32 is shown in Fig. 2, along with the 1970 September upper limit and with data from the 1–10 keV observations. While simple power law extrapolations of the high energy data seem generally consistent with those taken at lower energy, the absence of simultaneous measurements over the entire 1–300 keV range prevents us from determining whether spectral changes accompany the intensity variations. In any case, the fact that the spectrum extends to 300 keV, apparently without a break, sets 4U0041+32 apart both from the other hard transients and also from the hard compact binary X-ray sources, which generally have a spectral cut-off below a few tens of keV. The spectrum is similar to, but harder than, spectra of some compact extragalactic sources (for example, Cen A, NGC4151, and 3C273; see refs 14–16) and most resembles the spectra of certain cosmic γ -ray bursts (for example, 14 May 1972; see ref. 17). The source is distinguished from the other hard transients not only by its lack of a high energy spectral cut-off, but also by its position (>2.5 kpc below the galactic plane), by its apparent lack of pulsation⁵, and by not being associated with an early type giant or supergiant companion. Assuming a distance of 5 kpc, the peak X-ray luminosity of $\sim 3 \times 10^{37}$ erg s⁻¹ is in the range observed for other hard transients, and for the hard compact binary X-ray sources.

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Can pregalactic black holes produce the hard X-ray background?

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The possibility that pregalactic black hole accretion could have heated the Universe sufficiently to produce the hard X-ray background through bremsstrahlung emission is considered here. This would only be possible if the holes were implausibly large. However, the radiation produced directly from the accretion could explain both the density and spectrum of the X-ray background providing that the holes had a density of the order of 10^{-6} times the critical density and a mass of the order of $10^9 M_\odot$. We suggest that these holes could be the precursors of the holes which may presently be powering Seyfert galaxies or quasars.

It has been claimed¹ that the spectrum of the extragalactic diffuse X-ray background from 3 to 50 keV is well described as optically thin bremsstrahlung radiation with a temperature of ~ 45 keV. The energy density of this background, $\Omega_x \sim 10^{-8}$ in units of the critical density, could conceivably be generated by known discrete sources² (such as quasars). However, it would be surprising if the superposition of many discrete sources, each presumably having a different spectrum, could produce the very specific background spectrum required, and this suggests³ that the X-ray background may be produced by a uniform intergalactic medium with density $\Omega_g \sim 0.5$ and temperature 5×10^8 K. This would naturally explain why the background should have an optically thin bremsstrahlung spectrum. If such a medium does exist, however, it is hard to explain how it became so hot. Models which invoke quasar heating face severe energetic problems and, although these problems are alleviated if one assumes that the medium is clumped into large clouds, one would then expect anisotropies in the X-ray background larger than is observed⁴.

We first consider whether the intergalactic medium could have been heated to 5×10^8 K as a result of pregalactic black hole accretion. This suggestion is prompted by the realisation that black holes would have been accreting very rapidly at pregalactic epochs and that, if they produced radiation with an appreciable efficiency, they would have inevitably boosted the matter temperature well above the usual Friedmann value, generally causing reionisation. There are many contexts in which a population of black holes could have formed before galaxy formation. They could have been produced even before decoupling if the early Universe had large density fluctuations⁵ or a soft equation of state⁶ or it was highly anisotropic⁷ or cold⁸ or tepid⁹. More conventionally, one might expect holes to form after decoupling when the Jeans mass drops to $10^6 M_\odot$ and regions that size should bind very easily; such regions might either collapse directly or fragment into massive (10^2 – $10^4 M_\odot$) stars which could themselves collapse after their brief nuclear-burning phase of 10^6 yr. Within all these contexts one would expect the holes to have a mass above $10 M_\odot$ and their density Ω_B could, in principle, be close to 1. In this case, they would be good candidates for providing the 'missing' mass in clusters and galactic haloes¹⁰. If their density was very small, they might be identified with the black holes which are suspected of residing in quasars and active galactic nuclei¹¹.

The effect of pregalactic black-hole accretion on the thermal history of the Universe has been studied in detail elsewhere¹².

Here we outline the argument for determining the maximum temperature attained. We assume that the holes are subsonic at pregalactic epochs and that they accrete at the Bondi¹³ rate $\dot{M} \sim 10^{11} M^2 n T_4^{-3/2} \text{ g s}^{-1}$, where M is in solar masses and n and T_4 are the background particle density in cm^{-3} and temperature in units of 10^4 K. (For simplicity we assume a critical background density; in this case $n \sim 10^{-5} z^3$ for $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$.) If the accretion generates radiation with an efficiency ϵ , then holes larger than $10^3 \epsilon^{-1} M_\odot$ will be accreting at the Eddington limit for some period after decoupling and during this 'Eddington phase' we assume such holes radiate at the Eddington limit $L_{\text{ED}} \sim 10^{38} \text{ M erg s}^{-1}$.

We need to know how much of the radiation goes into heating the Universe and how much goes into the background. Photons emitted at a redshift z will go into Compton heating providing their energy exceeds about $10 z^{-3/2} \text{ MeV}$. Thus if most of the radiation is at an energy $E_{\text{max}} = 10 \eta \text{ keV}$, nearly all of it will go into Compton heating at redshifts exceeding $z_* \sim 10^2 \eta^{-2/3}$; the fraction doing so thereafter is just $f_c \sim (z/z_*)^{3/2}$. Photons with energy $\leq z^{1/2} \text{ keV}$ will go into photoionisation heating. If most of the emergent radiation is at an energy higher than this, the fraction which goes into photoionisation heating will be small; for a flat spectrum this fraction is $f_p \sim \eta^{-1} (z/10^3)^{1/2}$.

For even very small values of the black hole density Ω_B one can show that the photoionisation heating will cause the matter temperature to rise at some time after decoupling and eventually reach a value of $\sim 10^4$ K. At this point the ionisation will increase to a value close to 1 due to collisions. Because of the inverse Compton cooling of the 3 K background,

$$\Lambda_r \equiv -\left(\frac{dT}{dt}\right)_r \approx \frac{\sigma_T \rho_r T}{m_e c} \approx 10^{-19} T z^4 \text{ s}^{-1} \quad (1)$$

(where ρ_r is the 3 K radiation density, σ_T is the Thomson cross-section and m_e is the electron mass), T cannot initially rise above 10^4 K. However, it will do so when the Compton heating of the holes

$$\Lambda_c \equiv \left(\frac{dT}{dt}\right)_c \approx \frac{n_B L_{\text{ED}} f_c}{nk} \approx 10^{-3} \Omega_B \text{ K s}^{-1} \quad \text{for } z > z_* \quad (2)$$

(where n_B is the black hole number density) becomes comparable to Λ_r . This occurs at a redshift $z_c \sim 10^3 \Omega_B^{1/4}$. Thereafter T , determined by the balance of Λ_c and Λ_r , rises as z^4 until the accretion rate falls below the Eddington limit at a redshift which can be shown to be

$$z_{\text{ED}} \approx 10^{3.3} \Omega_B^{1/6} (M\epsilon)^{-1/9} \quad (3)$$

(This exceeds z_* for the values of Ω_B and M which are of interest.) After the Eddington phase, Λ_c is reduced and T rises as $z^{-2/5}$ for $z > z_*$ and falls as $z^{1/5}$ for $z < z_*$. At a redshift of the order of 10 the Compton heating time scale falls below the expansion time scale, so T begins to fall faster. However, at about this time the thermal history may be affected by various postgalactic processes (such as quasar heating³), so that our analysis breaks down.

These considerations imply that the maximum temperature is attained at the redshift z_* when f_c falls below 1 and it is

$$T_{\text{max}} \approx 10^{3.2} (\Omega_B M \epsilon)^{2/5} \eta^{4/15} \text{ K} \quad (4)$$

providing this does not exceed the temperature of the hottest accretion-generated photons $\sim 10^8 \eta \text{ K}$. Our estimate of T_{max} is insensitive to the spectrum of the emitted radiation so long as most of the energy is at the upper end (that is, as long as the spectrum is shallower than E^{-1}). Clearly, T_{max} can only be as high as 10^8 K , say, if the holes are very large indeed. Even with the optimal assumptions ($\Omega_B \sim 1$, $\epsilon \sim 0.1$, $\eta \sim 10^3$) one requires M to exceed $10^{12} M_\odot$ and there are independent limits which preclude holes of this size from being very numerous¹⁰. It is,

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therefore, implausible that pregalactic black hole accretion could heat the Universe to the temperature required to explain the hard X-ray background, even though one would expect it to reionise the Universe ($T_{\max} > 10^4$ K).

We now consider whether the fraction of generated radiation which does not go into heating the Universe could explain the 45-keV background. After the redshift z_* , this fraction is essentially unity, whereas before z_* it is only $(z_*/z)^{3/2}$. It is easy to deduce¹² that the largest contribution to the background density derives from the epoch z_* and that the redshifted energy generated by each hole is

$$E(M) \approx (\epsilon M c^2 z^{-1} t)_* \approx 10^{46} \Omega_B^{-3/5} M^{7/5} \epsilon^{2/5} \eta^{-11/15} \text{ erg} \quad (7)$$

This corresponds to a present background density

$$\Omega_x \approx n_B E(M) / \rho_{\text{crit}} \approx 10^{-8} (\Omega_B M \epsilon)^{2/5} \eta^{-11/15} \quad (8)$$

The present temperature associated with this radiation, as $z_* \sim 10^2 \eta^{-2/3}$, is $E \sim E_{\max} z_*^{-1} \sim 10^2 \eta^{5/3}$ eV. Although equations (7) and (8) were derived previously¹², the 'energy' parameter η was there assumed to be arbitrary and epoch-independent, whereas, for any particular model of black hole accretion, the value of η may itself be determined by M and $\dot{M}$. In particular, for some disk accretion models¹⁴, there will be a band in the $(\dot{M}, M)$ plane in which one would expect to produce optically thin bremsstrahlung radiation with a well-defined temperature. From the Bondi accretion formula and equation (4), the accretion rate at the epoch z_* is

$$\dot{M} \approx 10^{13} \Omega_B^{-3/5} M^{7/5} \epsilon^{2/5} \eta_*^{-12/15} \text{ g s}^{-1} \quad (9)$$

and the associated 'disk' temperature¹⁴ is

$$\theta_* \equiv 10^8 \eta_* \approx 10 \left(\frac{\dot{M}}{M} \right)^{1/2} \text{ K} \approx 10^{7.5} \Omega_B^{-3/10} (M \epsilon)^{1/5} \eta_*^{-6/5} \text{ K} \quad (10)$$

where η_* specifies the value of η at z_* . One expects an optically thin bremsstrahlung spectrum providing θ_* is in the range 10^7 – 10^9 K.

We will not attempt here to investigate whether material accreted pregalactically would, in fact, form an accretion disk; this involves issues too complicated to discuss in the present context. However, note that the present hypothesis would almost certainly fail if the black holes did not form accretion disks. For example, were the accretion spherically symmetric, radiation could still be generated with an appreciable efficiency due to magneto-hydrodynamic dissipation¹⁵. However, because the dominant radiation mechanism would be synchrotron losses in this situation, one could not expect to produce optically thin bremsstrahlung radiation.

Equations (8) and (10) determine Ω_x and the present background temperature T_x in terms of Ω_B and M :

$$\Omega_x \approx 10^{-8} \Omega_B^{1/2} (M \epsilon)^{1/3}, \quad T_x \approx \theta_* z_*^{-1} \approx 10^6 \Omega_B^{-5/22} (M \epsilon)^{5/33} \text{ K} \quad (11)$$

Thus the temperature and (to a lesser extent) the density of the background are remarkably insensitive to the values of Ω_B and M . Indeed, for most hypotheses of pregalactic black hole formation, one would expect to produce a background of optically thin bremsstrahlung X rays, providing the holes accrete according to the disk mode. On the other hand, because the observed value for T_x is specified so precisely, one can still infer the necessary values of M and Ω_B to within an order of magnitude. The present temperature of the radiation, $T_x \sim 10^2 \eta_*^{5/3}$ eV, is 45 ± 5 keV (as required¹) providing $\eta_* = 40 \pm 3$, that is providing the accretion at z_* produces radiation with

energy $E_{\max} \approx 0.4$ MeV. In this case $z_* \sim 10$ and, because $\Omega_x \sim 10^{-8}$, equation (11) implies

$$\Omega_B \sim 10^{-6}, \quad M \epsilon \sim 10^8 \quad (12)$$

Values for Ω_B and $M \epsilon$ much different from these would fail to produce either the density or the temperature of the X-ray background. The largest reasonable value for ϵ is about 0.1; in this case one needs $M \sim 10^9$. Note that the radiation produced at pregalactic epochs before and after z_* will also contribute to the X-ray background at energies around kT_x . However, these contributions will be dominated by the radiation produced at z_* itself if the holes are then producing optically thin bremsstrahlung radiation.

In the above discussion we have assumed that the black holes have a unique mass, whereas one might expect them to span a range of masses. However, equation (11) shows that the dominant effect on Ω_x and T_x comes from the value of M which maximises the products $\Omega_B M^{2/3}$ and $\Omega_B^{-1} M^{2/3}$, respectively. For the anticipated¹⁰ types of mass spectra, the largest holes would have the biggest effect on both Ω_x and T_x , although they may not dominate the total black hole density. The largest holes would also determine the value of $T_{\max}$. With the favoured parameters ($\Omega_B \sim 10^{-6}$, $\epsilon M \sim 10^8$, $\eta_* \sim 40$), equation (4) implies that the pregalactic medium would have attained a maximum temperature $T_{\max} \sim 10^5$ K at a redshift of ~ 10 . The temperature of the intergalactic medium today would be somewhat different from this (depending on which postgalactic heating sources operate) and its density would depend on how much gas was left over after galaxy formation. It is interesting that recent estimates¹⁶ of the density and temperature of the intergalactic medium inferred from observations of Ly α absorption lines in quasars indicate that T was indeed about 10^5 K at $z \sim 2$.

Note that one cannot expect this picture to produce an exactly 45-keV bremsstrahlung spectrum, partly because the radiation comes from a range of redshifts and perhaps a range of black hole masses, but also because even an individual hole will not have a unique temperature. There will be a superposition of spectra from different points on the accretion disk. All one can state is that the dominant contribution may fit a 45-keV spectrum; a more detailed analysis would be needed to determine whether the distortion due to the lesser contributions would be within the error bars of the data. The important point is that the background conditions should be very uniform before galaxy formation, so one would not expect the range of spectra exhibited in postgalactic sources like quasars. Note that Barrow and Silk have also suggested that pregalactic black hole accretion could generate the X-ray background¹⁹. However, they do not attempt to fit the actual spectrum; nor do they allow for the effect of the accretion on the thermal history of the Universe.

Finally, we consider the radiation produced by the black holes during the present epoch. The production by holes which reside in galactic haloes or clusters has been estimated previously¹². Such holes might in principle generate the density of the background radiation, for suitable values of Ω_B and M , but one would not expect them to accrete at a rate sufficient to produce a 45-keV optically thin bremsstrahlung spectrum. For example, if one assumes reasonable values for n and T ($n \sim 10^{-3}$, $T \sim 10^6$ in haloes; $n \sim 10^{-3}$, $T \sim 10^8$ in clusters), then equation (10) requires $M \sim 10^9 M_\odot$ for halo holes and $M \sim 10^{14} M_\odot$ for cluster holes. Both these possibilities are precluded by observation¹⁰. Liang has considered the possibility that the holes are intergalactic¹⁷ and that the ambient conditions are there as inferred by Sargent *et al.*¹⁶. He argues that such holes could also produce the 45-keV background density. On the other hand, with the sort of values for Ω_B and M required, it is perhaps more plausible to envisage the holes—rather than inhabiting any of the environments discussed above—presently residing in galactic nuclei. For a large black hole would naturally act as a condensation nucleus for a galaxy¹⁸ and the sorts of holes which have been hypothesised to power quasars and Seyfert galaxies¹¹

would indeed have values for Ω_B and M of the order of those we have inferred. In this case one could identify the sources of the hard X-ray background as the precursors of the most prominent X-ray sources at the present epoch. This would please proponents of the view that known sources can produce the background if one allows for evolutionary effects.

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Possible transfer of lunar matter to Earth due to a nearby supernova

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Theories of supernova explosion (SNEs)¹⁻³ as well as correlation of NO_3^- in Antarctic ice cores with the dates of historical supernovae⁴, suggest that many Type I SNE may yield an intense 10^{50} erg emission of γ rays. Such a SNE may be expected to occur within a few tens of light years of our Solar System once every hundred million years or so^{5,6}. A short γ -ray pulse of this magnitude would not only directly affect the terrestrial atmosphere in ways that can be important for surface life⁵, but also be absorbed by the lunar surface. Depending on the γ -ray energies involved, the upper centimetre of lunar surface soil can be evaporated by heating or ejected by the emergence at the surface of a rising pressure pulse. Such expelled lunar soil, moving at speeds near the $2 \times 10^5 \text{ cm s}^{-1}$ lunar escape velocity, could be captured by the Earth's gravitational field and gradually settle through the atmosphere to the Earth's surface. Geological strata for that epoch might then be expected to show an overabundance of iridium (but probably less than that found by Alvarez *et al.*⁷ in 65-Myr-old sedimentary layers at Gubbio). This would occur since the expelled lunar surface soil should be characterised by an Ir abundance greatly enriched, relative to terrestrial of lunar rocks, by micrometeorite bombardment.

SNE models often involve a configuration in which the dense exploding stellar core is surrounded by a rather minimal atmosphere^{3,5}. Under such circumstances, the outgoing supernova shock wave propagating through a very steep density gradient will become extremely relativistic. The hot relativistically expanding matter at the shock front emits a burst of radiation when only one optical depth of matter remains to be penetrated. The interval over which this radiation pulse arrives is greatly compressed, due to the relativistic expansion of the radiating matter. Colgate originally estimated a γ -ray flash (with a total energy $\sim 10^{48}$ erg) compressed into a pulse of duration $\leq 10^{-5}$ s (refs 1, 5). There exists a great variation in estimated γ -ray energies, from $\sim$ few MeV upward, and in total energies of the pulse up to $\sim 10^{50}$ erg (refs 2, 3).

A recent analysis of NO_3^- concentrations in an Antarctic ice core revealed the presence of four spikes coincident with historically known supernovae⁴. Two of these are known to have been Type I supernovae; the other two are not identified. To explain the large magnitudes of the four NO_3^- spikes, it seemed that at least 10^{50} ergs of X rays or γ rays would have to be emitted by each SNE. This radiation could, however, have arrived over an interval as long as a year rather than as a flash.

No γ -ray flashes have yet been detected in coincidence with extragalactic SNEs. However, the γ -ray flash, which should arrive well before the SN is visually observed, may well be associated only with a specific subclass of Type I SNEs. Furthermore, if the characteristic energies of the γ -rays are very high, it is possible these bursts would have escaped detection: fairly massive detectors need to be flown in satellites to allow the full e^+ shower to develop. We note also that γ rays above $\sim 10^{15}$ eV will produce e^+ pairs in collisions with the universal 3 K black-body radiation photons. This limits the detectability of such γ -ray sources to those within about 10^4 light yr. γ -ray flashes from the historical galactic supernovae could then have reached us, but not those from explosions in other galaxies.

We shall next consider some consequences of an assumed 10^{50} erg γ -ray flash from a SNE at a distance of 30 light yr or less from our Solar System. This would give an incident γ -ray flux in excess of $\sim 10^{10} \text{ erg cm}^{-2}$ locally.

The Earth's atmosphere shields its surface from the direct impact of an intense X-ray or γ -ray pulse. Effects on the surface would be indirect, such as those resulting from NO production and subsequent O_3 destruction. In contrast, the lunar surface receives the full flux. The detailed consequences of this exposure depend somewhat on the regime of incident γ -ray energies.

MeV γ rays, for which Compton scattering is the main energy deposition mechanism, are absorbed by $\sim 1 \text{ g cm}^{-2}$ of lunar surface matter. Thus, $\sim 10^{10} \text{ erg g}^{-1}$ of rapidly absorbed energy will be deposited in the solid surface layer, which has a density of $\sim 1 \text{ g cm}^{-3}$, down to a depth of 1 cm. This will increase the temperature of the centimetre layer to 10^3 – 10^4 K and generally vaporise it, assuming the γ -ray energy is deposited in less than a second (for $T \sim 3 \times 10^3$ K). The kinetic energy required for escape from an isolated moon is also $\sim 10^{10} \text{ erg g}^{-1}$, roughly comparable to the energy provided by the absorbed γ -ray flash. (Because of the Earth's gravitational pull, an additional 40% is needed to escape the Earth–Moon system.) Therefore, the hot evaporated vapour, which temporarily provides the Moon with an atmosphere of density $\sim 10^{-12} \text{ g cm}^{-3}$, will expand to fill and ultimately overflow the Roche lobe. A substantial fraction of this material, flowing through the inner lagrangian point into the Earth's Roche lobe, should ultimately reach the Earth. It would initially form a ring, at a radius of the order of 10^{10} cm, in which the particles have roughly keplerian velocities. Because of the extremely low gas density, $\sim 10^{15} \text{ g cm}^{-3}$, the mean free path for molecular collisions is about 10^{10} cm, that is comparable to the ring dimension. This causes the gas to behave as an extremely viscous fluid. The keplerian differential rotation then causes the main part of the disk to contract on a rapid time scale, if the outer material can be dispersed. During the descent of this lunar matter to the Earth, it would be affected by the solar wind; however, the wind pressure $\sim 10^{-10} \text{ dyn cm}^{-2}$ on 10^{18} g of falling vapour is insufficient to sweep even fully ionised matter of this kind away from the Earth is less than 10 yr (M. Rees, personal communication). Neutral gas will survive for much longer. The accreted lunar gas may take several decades to move (mainly by advection) down through the upper atmosphere and stratosphere and ultimately reach the Earth's surface.

A somewhat different theory for an ejection of lunar surface matter would be required if the SNE γ -ray flash were characterised by photon energies $\gg 100$ MeV, arriving within an interval of the order of 10^{-5} s. For these conditions, the incident γ -ray energy is deposited at a depth of a few tens of cm, mainly by ionisation resulting from an electron–positron shower which develops over several radiation lengths (l). If the duration of the γ -ray pulse is $\leq 10^{-4}$ s (l over the local sound speed), the energy

is deposited on a time scale less than that for thermal expansion in response to the initial differential heating. The resulting initial pressure excess, the product of the temperature rise, the coefficient of thermal expansion, and the volume compression modulus, is of the order of $10^{10} \text{ l}^{-1} \sim 10^9 \text{ dyn cm}^{-2}$ within the main energy deposition layer. Emergence of this pressure impulse at the lunar surface which has weak or negligible tensile strength should result in mass ejection. Matter ejected at the speed of sound in lunar matter has about the 10^5 cm s^{-1} speed needed to escape from the Moon and still be captured by the Earth.

If a SNE γ -ray flash and the consequent transfer of lunar material to the Earth occurred $>60 \text{ Myr}$ ago, it would be difficult to find evidence of it on the surfaces of lunar rocks today. Micrometeorite bombardment erodes those surfaces at the rate of $10^{-1} \text{ cm Myr}^{-1}$ (ref. 8), so that direct evidence for or against melting and recrystallisation or fragmentation of the outer centimetre or so of lunar surface matter is unlikely (G. Turner, personal communication). (Some evidence for such a flash might still be found in the form of glassy fragments, which were buried before they could be eroded.) The lunar samples studied have also come from a relatively small region, about 10^3 km across, which would have one chance in two of having been on the dark side of the flash.

If a strong $10^{10} \text{ erg cm}^{-2}$ γ -ray flash is likely every 10^8 years from SNEs uniformly distributed in space and time, a flash of strength $10^{10} \text{ f erg cm}^{-2}$ would be expected to have occurred as recently as $10^8 \text{ f}^{3/2} \text{ yr}$ ago. Thus, about 10^6 years ago there might have been a final γ -flash of intensity $5 \times 10^8 \text{ erg cm}^{-2}$. This would not have been intense enough to evaporate lunar surface matter but could have melted almost 1 g cm^{-2} of it, that is, to a depth of a few millimetres if the flash γ rays had energies in the X-ray to MeV region. Erosion would not yet have removed all of the melted-recrystallised surfaces. One is tempted to speculate that such a flash might still be in fact observable in the form of the glassy patches emphasised by Gold⁹, who proposed a sudden (solar) flash for the original melting. On the other hand, a relatively nearby SNE may be 'common' only when the Solar System is within a spiral arm, for example, of the order of 60 Myr ago. A SNE γ -ray flash giving $5 \times 10^8 \text{ erg cm}^{-2}$ some 10^6 years ago would then be less likely.

A possible consequence of the transfer of $\sim 1 \text{ g cm}^{-2}$ of the lunar surface to that of the Earth rapidly enough to be, in effect, instantaneous over geologically measurable intervals, is element abundance anomalies at appropriate places in the terrestrial sedimentary layers. Alvarez *et al.*⁷ report a large enhancement in the abundance of Ir, from $0.25 \times 10^9 \text{ g per g}$ of limestone to $6.35 \times 10^{-9} \text{ g per g}$ in a 1-cm layer at Gubbio. This anomaly is identified as occurring at the Cretaceous-Tertiary boundary about 65 Myr ago. The isotope abundance ratio remained normal.

The Earth's surface is deficient in Ir relative to the meteorites by a factor of more than 10^3 . While lunar rocks are also characterised by a large deficiency, the upper centimetre of lunar soil, which is strongly adulterated by micro-meteorite bombardment, can be expected to show significant Ir concentrations. Analyses of Apollo 11 and Apollo 12 lunar soil samples^{10,11} reveal Ir abundances up to $\sim 10 \times 10^{-9} \text{ g per g}$; this represents typically an average over the upper few metres or so of the regolith. After a study of the overall character of the trace-element abundance patterns, this enrichment has been attributed^{10,11} to the addition of carbonaceous chondrite-like material at a rate of the order of $4 \times 10^{-9} \text{ g cm}^{-2} \text{ yr}^{-1}$ over $4.5 \times 10^9 \text{ yr}$. (The Ir concentration in chondrites is over 1,000 times that found in the crust of the Earth and 100 times that of lunar soil.) At this rate, 0.4 g cm^{-2} of the meteoritic material would be accumulated in the 10^8 year intervals between our hypothesised occurrences of intense γ -ray flashes. The rapid transfer of this amount of meteoritic matter alone to the surface of the Earth would provide 0.03 g cm^{-2} which, when mixed into 1 g cm^{-2} of terrestrial soil, would yield an Ir concentration of about $15 \times 10^{-9} \text{ g per g}$. However, turnover of lunar surface

matter occurs at a rate given approximately by 0.1 cm Myr^{-1} (ref. 8). If there were mixing to a depth of several centimetres over a 10^8 year interval, the average Ir content of this surface material would be of the order of $3 \times 10^{-8} \text{ g per g}$. There is no compelling reasons for the lunar surface to be homogeneous: some patches may have more than this while most probably have less. The Ir results obtained by Alvarez *et al.*⁷ might be understood in this way only if the top several g cm^{-2} of lunar soil were evaporated and now contributed the measured Ir at the appropriate geological stratum. Alternatively, we might assume the top g cm^{-2} has a significantly higher average value, $\sim 10^{-7} \text{ g per g}$, since there might be large patches which do not undergo such thorough mixing before evaporation. If most of this material reached the Earth and mixed with 1 g cm^{-2} , the Ir discontinuity obtained at Gubbio could again follow, but its magnitude does seem much larger than is easily explained by lunar surface matter transfer.

Orbits of near Earth micrometeoritic particles will be strongly altered by non-symmetric surface evaporation resulting from a γ -ray flash. This too may cause a temporary increase in the fall of very Ir rich matter onto the Earth (A. Fabian, personal communication).

A $10^{10} \text{ erg cm}^{-2}$ flash would lead directly to very strong ozone suppression in the Earth's atmosphere, and thus very greatly increased solar UV radiation at the Earth's surface for about a decade⁵. In addition, during the long period of lunar soil mass transfer through our atmosphere, the transparency of the dirtied terrestrial atmosphere to visible and Ir radiation would be very greatly altered, even if the lunar matter remained mainly gaseous. The added lunar matter could be almost a tenth as abundant as atmospheric CO_2 . If the lunar matter were dustlike, the direct atmospheric albedo together with changed cloud seeding would significantly influence the surface climate.

The detailed consequences of these various possible environmental modifications on biological species are not known. It seems reasonable to speculate that a biological cataclysm may have resulted. A key question is whether or not the great extinctions at the end of the Cretaceous age in which approximately one-quarter of all species, including the dinosaurs, died were sufficiently simultaneous¹². Some recent data incorporating times of magnetic field reversals have been interpreted as indicating that the faunal overturn at Gubbio took $<10^4 \text{ yr}$ (ref. 13) and that the Foraminifera and dinosaur extinctions in Alberta were 'simultaneous' with a maximum error of 10^5 yr (ref. 14). While such data hardly insist on a sudden catastrophic explanation, they do question the notion that there are no strong correlations among the extinctions or that they are all caused by slowly changing geological conditions.

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Reactions of OH radicals with gaseous sulphur compounds

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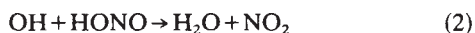
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Several recent papers¹⁻³ have discussed the origin of the sulphate aerosol in the stratosphere in terms of atmospheric carbonyl sulphide (OCS) and carbon disulphide (CS₂). There is, however, considerable uncertainty in the life cycles and budgets of these compounds. This arises from a lack of knowledge of the various possible sources and also from the differences in the reported rate constants for the reactions of OCS and CS₂ with hydroxyl (OH) radicals^{4,5}, which provide a potentially large sink for these molecules in the troposphere. We report here measurements of rate constants and product formation for OH reaction with several sulphur compounds, including OCS and CS₂. The results support the suggestion that oxidation of CS₂ is a significant source of atmospheric OCS, but do not give conclusive evidence for a large sink for OCS in the troposphere.

Interest in atmospheric sulphur chemistry has recently focused on the sulphate component in the stratospheric aerosol layer which plays a significant role in the Earth's radiation budget. In addition to direct injection of SO₂ into the stratosphere from volcanic activity, an important role for OCS as a carrier of sulphur from the troposphere to the stratosphere has been suggested¹. Recent measurements⁶⁻⁹ show that OCS is indeed widely distributed in the atmosphere. Photolysis of OCS in the stratosphere releases atomic S which is oxidised and subsequently converted to H₂SO₄ aerosol. It has also been proposed that reaction of OCS with OH is the origin of the SO₂ present in the background troposphere¹⁰ and that a similar reaction of CS₂ is a source of atmospheric OCS as well as SO₂ (ref. 2).

In the present work rate constants were measured using a competitive rate technique, in pseudo-atmospheric conditions. Hydroxyl radicals were generated by the photolysis at 350 nm of ~5 p.p.m. of nitrous acid (HONO) in synthetic air at 1 atm pressure and ambient temperature (297 ± 2K), contained in a 200 l bag fabricated of Tedlar film. Photolysis of the mixture results in a steady state concentration of OH radicals which at small conversion is governed by the reactions (1) and (2)¹¹



[OH] can be conveniently monitored in this system by addition of a few p.p.m. of ethylene (C₂H₄) which, following attack by OH, is oxidised to two molecules of formaldehyde by a chain reaction in which OH is regenerated^{12,13}. By monitoring the rate of C₂H₄ decay, [OH] can be calculated from the relationship

$$-\frac{d \ln [\text{C}_2\text{H}_4]}{dt} = k_3 [\text{OH}] \quad (3)$$

k_3 is the rate constant for the reaction of OH + C₂H₄ which at atmospheric pressure has a value of $8 \times 10^{-12} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$

(ref. 14). Rate constants for the reaction of OH with the sulphur compounds were obtained relative to k_3 , by measurement of the decay of the sulphur compound and of C₂H₄ during photolysis in the HONO-Air mixtures for ~30 min. Expressing the integrated forms of equation (3) as ratios for the two substrates gives

$$\ln \frac{[\text{S}]_0}{[\text{S}]_t} = \frac{k_s}{k_3} \ln \frac{[\text{C}_2\text{H}_4]_0}{[\text{C}_2\text{H}_4]_t} \quad (4)$$

where the subscripts refer to concentrations at time 0 and t ; k_s is the rate constant for reaction of OH with the sulphur compound, S.

Ethylene, methyl mercaptan (MM), dimethylsulphide (DMS), dimethyldisulphide (DMDS) were all measured by gas chromatography using flame ionisation detection. H₂S, SO₂, OCS and CS₂ were measured using a flame photometric detector after separation on a 1 m Chromasil 316 column mounted in an all-PTFE chromatographic system. HONO, NO and NO₂ were measured using a commercial chemiluminescence detector. All gases and vapours were used as supplied commercially except for OCS from which H₂S impurity was removed to a level of <0.01% by passage through aqueous Cu(NO₃)₂.

Figure 1 shows a plot of the concentration-time data according to equation (4). Good straight line plots were obtained in the main, indicating that the removal of both substrates was dominated by reaction with OH. For CS₂ and DMS there was a tendency for their removal rates to increase relative to C₂H₄ later in the reaction. This can be attributed in part to reaction with oxygen atoms, which are produced from photolysis of NO₂ formed during photolysis. Also CS₂ absorbs light in the 300–350 nm region; in the absence of HONO, the decay rate of CS₂ was a factor of 3 slower indicating that although direct photolysis was significant in the HONO-CS₂ system, it was not the major decay route for CS₂.

Table 1 Rate constants for OH + sulphur compounds

Gas	$\frac{k_s}{k_{\text{C}_2\text{H}_4}}$	$k_s (\text{cm}^3 \text{ molecule}^{-1} \text{ s}^{-1} \times 10^{-12})^*$
DMDS	28 ± 10	223 ± 80
MM	11.3 ± 1.1	90.4 ± 8.5
DMS	1.14 ± 0.18	9.1 ± 1.4
H ₂ S	0.62 ± 0.04	5.0 ± 0.3
SO ₂	0.09 ± 0.02	0.72 ± 0.16
CS ₂	0.06 ± 0.02	0.43 ± 0.16
OCS	≤ 0.005	≤ 4 × 10 ⁻²

* Using $k_{\text{C}_2\text{H}_4} = 8 \times 10^{-12} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$.

Table 1 shows a summary of the rate constant data from the plots according to equation (4). The OH rate constants are in good agreement with previous results for SO₂ (at 1 atm pressure)¹⁵, H₂S (refs 15, 16) and DMS (ref. 14). A value for k (OH + DMDS) has not apparently been reported before. For both CS₂ and MM the values are both higher than measured directly using the resonance fluorescence technique at low total pressures^{4,5,14}. We conclude that the reaction of OH with MM is enhanced at high pressure. The position for CS₂ is less well defined; the CS₂ value given has been corrected for the apparent contribution of ~40% to the CS₂ decay, due to photolysis and O(³P) reaction, but the possibility of a systematic error in this determination cannot be entirely ruled out. We are, therefore, unable to conclude definitely whether there is a pressure effect on k (OH + CS₂) but our results are certainly more consistent with the value reported by Kurylo⁵ that is, $1.8 \times 10^{-13} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ than with the upper limit value of Atkinson *et al.*⁴, $7 \times 10^{-14} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$.

For OCS, the absence of any measurable decay gave an upper limit comparable with the largest reported value for k (OH +

OCS (ref. 5), that is, $5.66 \times 10^{-14} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$. However, observation of the effect of a large excess of OCS on the apparent [OH] calculated from equation (3) in the HONO- C_2H_4 system, indicated that the true value of $k(\text{OH} + \text{OCS})$ may be considerably lower than this upper limit. A value of $\sim 8 \times 10^{-15} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ could be derived from the data but uncertainties in the detailed chemistry precluded an accurate determination of this rate constant.

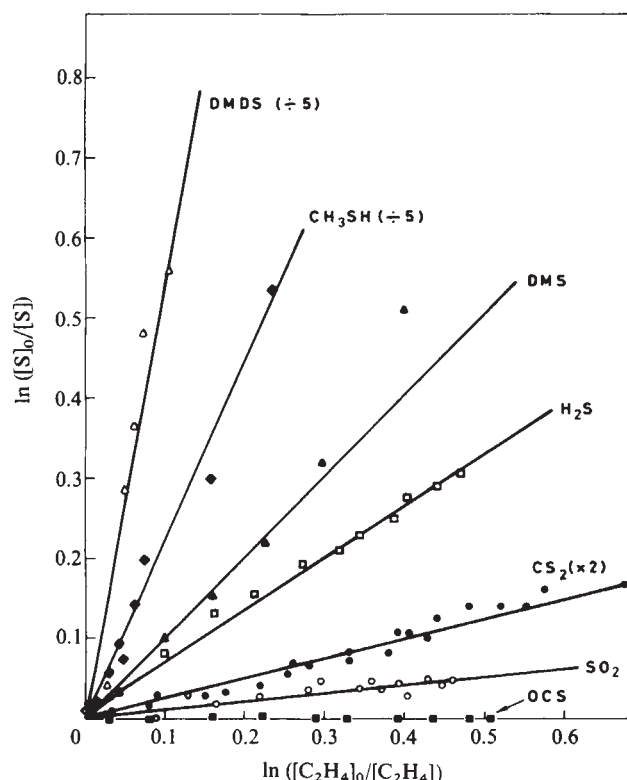
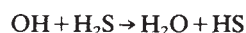
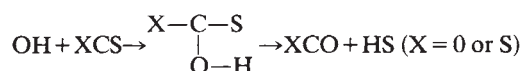


Fig. 1 Plot of $\ln [S]_0/[S]$, versus $\ln [C_2H_4]_0/[C_2H_4]$, for the decay of sulphur compounds and ethylene during photolysis in HONO/air mixtures at atmospheric pressure and 297 K.

Investigation of product formation in these systems revealed that SO_2 is a major product in the reaction of OH with all the S compounds investigated, with the exception of SO_2 itself which is oxidised to an H_2SO_4 aerosol¹⁷. H_2S was converted quantitatively to SO_2 and, as the initial attack of OH with H_2S undisputably occurs through the reaction



we conclude that HS is efficiently converted to SO_2 in our experimental conditions. The observation of SO_2 formation in the $\text{OH} + \text{CS}_2$ and $\text{OH} + \text{OCS}$ system therefore supports the suggestion⁵ that these reactions proceed through an addition complex, which yields HS as a primary product:



Further indication that this is a major pathway for CS_2 comes from the observation of the production of OCS with a high yield ($\sim 100\%$) in the $\text{OH} + \text{CS}_2$ system.

Note the significance of the results in the context of the behaviour of CS_2 , OCS and the organic sulphides in the global

sulphur cycle. The ubiquitous presence of OH in the troposphere provides a sink mechanism for gases with which it reacts. A relationship between tropospheric lifetime and OH reaction rates has been computed using a two-dimensional model¹⁸; our value of $k(\text{OH} + \text{CS}_2)$, that is, $4.3 \times 10^{-13} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ gives a lifetime of 0.2 yr for CS_2 .

Our results show that the reaction of CS_2 with OH radicals provides a potentially large source of OCS in the troposphere. The major uncertainty in the calculation of the source strength is due to the paucity of observational data for atmospheric CS_2 . The only reported measurements of CS_2 in air⁷, gave concentrations in the range 0.07–0.37 p.p.b. (parts per 10^9). Subsequent measurements have confirmed that CS_2 concentrations near the lower end of this range are normally present in the atmosphere at Harwell (S. A. Penkett, personal communication). If the value of 0.07 p.p.b. is taken to be representative of background tropospheric air, the source strength of OCS corresponding to a lifetime of 0.2 yr is $2 \times 10^{12} \text{ g(S) yr}^{-1}$. This is considerably larger than estimates of OCS emissions from identified sources, that is, biomass burning, $2.4 \times 10^{11} \text{ g(S) yr}^{-1}$ (ref. 19), soil emissions $2.3 \times 10^{11} \text{ g(S) yr}^{-1}$ (based on flux measurements in the US²⁰).

The source of OCS from CS_2 alone is considerably larger than estimates of the amount of OCS removed by photolysis in the stratosphere^{1,2}, $(2-5) \times 10^{10} \text{ g(S) yr}^{-1}$, and implies a large sink for OCS in the troposphere. Measurements⁶⁻⁹ of tropospheric OCS have shown that it is fairly uniformly distributed with a concentration of about 0.4 ± 0.1 p.p.b., and, to balance the source from CS_2 , would require a lifetime of 1.1 yr for OCS. A sink of this magnitude could be provided by reaction with OH only if the 'high' value for the rate constant $k(\text{OH} + \text{OCS})$ (ref. 5) was correct. However, the true value may be lower by up to a factor of 10, which suggests that an additional sink for OCS may be operative. Physical removal of OCS on vegetation²¹ or at ocean surfaces may fulfil this role.

The reaction of OH with CS_2 as formulated above also provides a source of SO_2 , equivalent in size to that of OCS. In this case, however, the source is small compared with the global flux of sulphur in the troposphere $1 \times 10^{14} \text{ g(S) yr}^{-1}$ (ref. 22) of which about 60% is due to SO_2 emission from fossil fuel combustion. The remainder of the tropospheric sulphur is thought to originate from oxidation of H_2S , DMS and other organic sulphur compounds, primarily of natural origin. The present work confirms their short tropospheric lifetime due to rapid reaction with OH, and also shows that SO_2 is an important product of these reactions in pseudo-atmospheric conditions.

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Enrichment of ^{210}Pb and ^{210}Po in the sea-surface microlayer

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Chemical fractionation at the air-sea interface is frequently suggested¹⁻⁵ as a possible mechanism contributing to the enrichment of a number of trace metals in the marine atmosphere^{6,7}. The origin of the long-lived radon daughters (^{210}Pb , ^{210}Bi and ^{210}Po) in the atmosphere is of special interest because of their use in the estimation of tropospheric aerosol residence times⁸. It has been proposed⁹ that injection of a fractionated marine component by bubbles bursting at the sea surface might be responsible for the occurrence of anomalously high atmospheric $^{210}\text{Po}/^{210}\text{Pb}$ ratios in Antarctica¹⁰ and Hawaii¹¹. Here we report the first measurements of ^{210}Pb ($t_{1/2} = 22.3$ yr) and ^{210}Po ($t_{1/2} = 138$ d) in the sea-surface microlayer. These measurements were made to determine whether or not a significant flux of these radionuclides from the sea surface to the atmosphere could occur. We also introduce a method for treating chemical data that we believe may be useful in determining the origin of the trace-metal enrichments often observed^{1,2} in the microlayer.

The sea-surface microlayer is the uppermost thin skin of the oceanic water column. It is often characterised by anomalously high concentrations of heavy metals and other trace substances thought to be associated with particulate matter and surface-active organic material^{1,2}. The thickness of the microlayer is defined by the method used to sample it. We used a plastic screen in a modification of the technique of Garrett which samples the upper 300 ± 50 μm (refs 12, 13). Because this was an initial attempt to detect enrichments of ^{210}Pb and ^{210}Po in the microlayer, we deliberately chose to collect our screen samples in calm sea conditions when visible surface slicks were present. Bulk surface-water samples were collected by immersing polyethylene jugs to a depth of about 20 cm and allowing them to fill. We also analysed a single sample of foam collected along the shore of a coastal pond. This material is believed to represent a more concentrated example of the anomalously enriched layer than samples provided by the screen technique^{14,15}. Details of the analytical procedures are given elsewhere¹⁶.

Results are shown in Table 1. In all cases both ^{210}Pb and ^{210}Po showed enrichments in the sea-surface microlayer samples in comparison with bulk surface-water samples. The degree of enrichment seemed to correlate with film pressure (depression of surface tension) estimated by the oil-drop method^{13,17}, although film pressures were always near the limit of detection. The greatest enrichments occurred in the foam sample from Oyster Pond. Thus a correlation exists between enrichments of the radionuclides and the presence of surface-active material.

To assess the role of the sea surface in supplying metal-rich aerosols, one must determine whether the enrichments in the microlayer are maintained by deposition of atmospheric material or by concentration of the metals from seawater. Only in the latter case can the sea act as a source. The $^{210}\text{Po}/^{210}\text{Pb}$ activity ratio may provide an important clue. Table 2 lists representative values of the $^{210}\text{Po}/^{210}\text{Pb}$ ratio in atmospheric fallout and in seawater and marine particulate matter from both coastal and open-ocean environments. The atmospheric ratio is much lower than the ratios in bulk surface seawater measured in this study, yet the $^{210}\text{Po}/^{210}\text{Pb}$ ratios in the microlayer samples show only small departures from the bulk seawater values. This comparison suggests that the ocean may be an important source of the enrichments found in the microlayer.

A more quantitative treatment of this question can be made by consideration of a simple model in which the surface-microlayer enrichment consists of two components: an atmospheric component and an oceanic component supplied by concentration from seawater and upward transport to the air-water interface. This latter supply process may involve chemical fractionation. It is assumed that processes removing material from the microlayer, either physical injection into the atmosphere or mixing downward in the water column, involve no chemical fractionation. With this assumption we can write for ^{210}Pb :

$$\Delta_{\text{Pb}} = \text{Pb}_m - \text{Pb}_b = \epsilon_{\text{Pb}}\text{Pb}_b + \text{Pb}_a \quad (1)$$

where the ^{210}Pb enrichment, Δ_{Pb} , is the difference between the ^{210}Pb concentration in the microlayer, Pb_m , and the concentration in bulk surface seawater, Pb_b . This concentration difference consists of an enriched oceanic component, $\epsilon_{\text{Pb}}\text{Pb}_b$, and an atmospheric component, Pb_a . A similar equation can be written for ^{210}Po :

$$\Delta_{\text{Po}} = \text{Po}_m - \text{Po}_b = \epsilon_{\text{Po}}\text{Po}_b + \text{Po}_a \quad (2)$$

Finally we assume that the atmospheric $^{210}\text{Po}/^{210}\text{Pb}$ ratio, R_a , is known:

$$R_a = \text{Po}_a/\text{Pb}_a \quad (3)$$

Equations (1)–(3) contain four unknown quantities, so it is not possible to solve them uniquely. It is possible, however, to derive a relationship between two important quantities. Let α denote the fractionation between ^{210}Pb and ^{210}Po during their transport from bulk seawater to the microlayer. This fractionation is given by

$$\alpha = \epsilon_{\text{Po}}/\epsilon_{\text{Pb}} \quad (4)$$

so if $\alpha > 1$, then ^{210}Po is preferentially (relative to ^{210}Pb) transported to the microlayer. This condition is probably necessary if the sea surface is to act as a source for the anomalously high $^{210}\text{Po}/^{210}\text{Pb}$ ratios in Antarctic aerosols¹⁰, because $^{210}\text{Po}/^{210}\text{Pb} < 1$ in most ocean surface water. (Coastal waters in Vineyard Sound (Table 1) and other locations^{18,19} are often the exception with $^{210}\text{Po}/^{210}\text{Pb} > 1$. The origin of the unsupported ^{210}Po in these waters is not understood. Over most of the ocean surface,

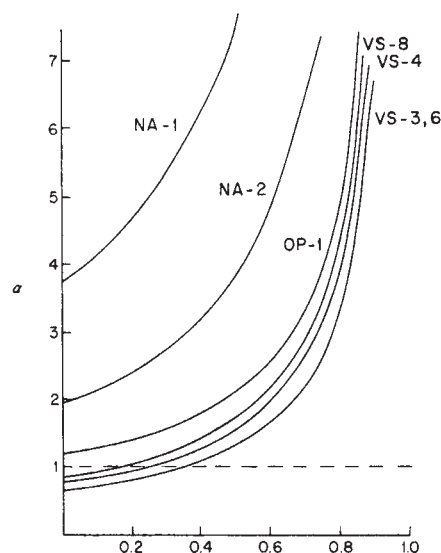


Fig. 1 Results of the model calculations for $R_a = 0.1$. Each screen sample is represented by a curve. In those cases for which more than one bulk surface-water sample was collected on the same date and in the same location, the results from Table 1 were averaged. For $\alpha = 1$ there is no chemical fractionation. For $f = 1$ all of the enrichment of ^{210}Pb in the microlayer is contributed from the atmosphere. The curves are fairly insensitive to variations of R_a in the range 0–0.2.

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Table 1 Analytical results*

Sample	Type	Description of sampling conditions	Film pressure† (dyn cm ⁻¹)	²¹⁰ Pb (d.p.m. per 100 kg)	²¹⁰ Po	Activity ratio ²¹⁰ Po/ ²¹⁰ Pb	ΔPo/ΔPb
Vineyard Sound, Massachusetts (14 July 1977)							
VS-1	Bulk	—	—	3.5±0.1	9.6±0.4	2.8±0.2	—
VS-2	Bulk	—	—	1.8±0.1	6.8±0.3	3.8±0.3	—
VS-3	Screen	Visible surface slicks, deliberately sampled	>1	12.5±0.3	29.6±0.9	2.4±0.1	2.2
VS-4	Screen	Slicks present but avoided	~1	6.6±0.3	18.3±0.6	2.8±0.1	2.6
Vineyard Sound, Massachusetts (5 September 1977)							
VS-5	Bulk	—	—	5.0±0.2	7.0±0.4	1.41±0.09	—
VS-6	Screen	Visible slick, some quantities of foam sampled	>1	41.3±1.6	51.5±1.9	1.25±0.06	1.2
VS-7	Bulk	—	—	3.2±0.1	7.0±0.7	2.2±0.2	—
VS-8	Screen	Patches of slick present but avoided	<1	6.0±0.2	10.0±0.5	1.7±0.1	1.6
Oyster Pond, Falmouth, Massachusetts (29 September 1977)							
OP-1	Foam	—	—	1,360±30	430±50	0.31±0.04	0.32
OP-2	Bulk	—	—	36.8±1.0	9.6±0.7	0.26±0.02	—
North Atlantic Ocean 45°50'N, 64°10'W (16 September 1977)							
NA-1	Screen	Patches of slick present, sample gathered both in and out of slick	~1	16.4±0.3	15.0±0.7	0.91±0.05	2.6
NA-2	Screen	Patches of slick present, sample gathered both in and out of slick	~1	21.3±0.7	21.7±1.2	1.02±0.06	1.7
NA-3	Bulk	—	—	14.7±0.5	10.5±0.6	0.71±0.05	—

* Uncertainties listed are standard errors based on counting statistics.

† These are approximate values. All measurements were near the detection limit, and there was often some variation in film pressure during each sampling period because of slick patchiness.

however, $^{210}\text{Po}/^{210}\text{Pb} < 1$ because of biological uptake and transport of ^{210}Po to deeper water²⁰.) We now let f denote the fraction of the microlayer ^{210}Pb enrichment that is contributed from the atmosphere, and we can write

$$f = \text{Pb}_a / (\varepsilon_{\text{Pb}} \text{Pb}_b + \text{Pb}_a) \quad (5)$$

With these definitions and with equations (1)–(3), we derive the following equation:

$$\alpha = (\Delta_{\text{Po}}/\Delta_{\text{Pb}} - fR_b) / (1 - f)R_b \quad (6)$$

where $R_b = \text{Po}_b/\text{Pb}_b$. Thus for any microlayer/bulk-seawater sample pair we can define a relationship between α and f . Note that this relationship is independent of the degree of dilution of the microlayer sample by bulk seawater.

Figure 1 shows curves based on equation (6) and the data in Table 1. Although our data do not allow a unique solution, the curves in Fig. 1 allow some limitations to be placed on their interpretation. We note that as f approaches a value of 1.0 (no oceanic contribution), α increases very rapidly. In other words, the more dominant the atmospheric contribution to the microlayer enrichment of ^{210}Pb becomes, the more strongly fractionated the oceanic contribution must be to compensate for the low $^{210}\text{Po}/^{210}\text{Pb}$ ratio in the atmospheric component. We believe that a reasonable interpretation of the data requires that at least part of the microlayer enrichments be supplied by concentration of ^{210}Pb and ^{210}Po from the bulk seawater.

At the other extreme, by setting $f = 0$ (no contribution from the atmosphere), we obtain the minimum possible value for α . To explain our open-ocean results (samples NA-1 and NA-2), we require $\alpha > 1$ no matter how small a value is assumed for f ; that is, concentration of ^{210}Pb and ^{210}Po from the bulk seawater must involve chemical fractionation such that ^{210}Po is preferentially transported to the interface. For the coastal water samples (sample OP-1 and the VS samples), $\alpha \approx 1$ (little or no fractionation) if no atmospheric contribution is assumed. This reflects the similarity of $^{210}\text{Po}/^{210}\text{Pb}$ ratios in microlayer and bulk surface-water samples at the coastal sampling sites. Only for $f \geq 0.4$ must significant fractionation be postulated.

Experiments in which columns of seawater are bubbled with air suggest that trace-metal enrichments in the microlayer may be maintained by transport of particulate matter to the interface⁵. If this were the predominant mechanism for ^{210}Pb and ^{210}Po , and if it acted indiscriminately for all types of particulate matter, then it would be possible to estimate α from the known distribution of ^{210}Pb and ^{210}Po between dissolved and particulate forms in surface seawater by using the expression

$$\alpha = (^{210}\text{Po}/^{210}\text{Pb})_{\text{particulate matter}} / (^{210}\text{Po}/^{210}\text{Pb})_{\text{bulk seawater}} \quad (7)$$

In open-ocean surface water^{21,22} this quantity is in the range 2.4–8.8 (median 6.7). Coastal surface waters (ref. 18 and unpublished data) are characterised by values closer to 1.0, probably because of the contribution of resuspended bottom sediment to the particulate matter. This difference may account for the segregation of our open-ocean samples from our coastal samples (Fig. 1), but further testing of this hypothesis is required.

We have shown that the sea-surface microlayer is enriched in ^{210}Pb and ^{210}Po . Our interpretation of the data suggests that a significant fraction of the enrichment is maintained by concentration from the bulk surface seawater. In the open ocean this concentration process results in preferential transport of ^{210}Po to the air-sea interface. In coastal environments there may be a

Table 2 Representative values of the $^{210}\text{Po}/^{210}\text{Pb}$ activity ratio in seawater, marine particulate matter and atmospheric fallout

Material	Median	Range	Refs
Surface North Atlantic			21, 22
Seawater	0.52	0.08–1.09	
Particulate matter	4.6	0.9–5.3	
Long Island Sound, US			18
Water (total)	0.89	0.80–1.44	
35 µm Seston	1.3	1.2–2.4	
333 µm Seston	12	1.4–62	
Atmospheric fallout	0.03	~0–0.16	18
New Haven, Connecticut			

lesser degree of chemical fractionation. Because coastal waters often have $^{210}\text{Po}/^{210}\text{Pb}$ activity ratios > 1 , the sea surface is, in either case, a potential source of aerosols with $^{210}\text{Po}/^{210}\text{Pb} > 1$ as suggested by Turekian *et al.*⁹. The natural flux of microlayer material from the sea to the atmosphere, however, is not known.

Note finally that our analytical treatment of the microlayer enrichments was limited because only two elements were used. Further analysis by a multi-element approach is suggested.

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Self-reversal of natural remanent magnetisation in the Olby-Laschamp lavas

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In 1967 Bonhommet and Babkine¹ reported reversed magnetisation directions in two lavas at Laschamp and Olby (Chaîne des Puys, Auvergne, France). They argued that the remanent magnetisation might have originated either from an episode of reversed polarity of the geomagnetic field within the Brunhes normal epoch or from a process of self-reversal² occurring in these lavas. I report here experimental evidence that the natural remanent magnetisation (NRM) of certain samples of the Olby flow undergoes complete self-reversal during thermal demagnetisation. Other Olby samples show partial self-reversal of NRM at room temperature.

Radiometric dating initially gave age limits to the extrusion of the Laschamp and Olby basalts between 8,000 yr and 20,000 yr BP (ref. 3) and since then the reversed volcanics have been generally accepted as a well documented case of a recent excursion of the Earth's magnetic field⁴, named the 'Laschamp geomagnetic polarity event'. Although Denham and Cox⁵ proved that it was highly probable that the geomagnetic field remained constantly normal on a global scale between 13,300 and 30,400 yr BP, many authors^{6–11} tried to correlate other events such as the Gothenburg⁶, Gulf of Mexico⁷, Maelifell¹⁰, Eriau¹¹ excursions with the Laschamp reversal. Age dating techniques have recently been refined. As a result of new K–Ar, ⁴⁰Ar/³⁹Ar, ²³⁰Th/²³⁸U and thermoluminescence dates^{4,12–14} it

seems likely that the true age of the 'Laschamp event' is between 40,000 and 45,000 yr BP. Consequently magnetic correlation is now being made with somewhat older reported excursions, such as the Lake Mungo¹⁵ or Lake Biwa¹⁶ records.

Whatever the significance of these correlation attempts, the importance of geomagnetic events or excursions as magneto-stratigraphic marker horizons is unquestioned. Nevertheless, I would like to put forward an alternative explanation for the Laschamp event. Thermomagnetic experiments performed on the reversely magnetised Olby flow demonstrate that a self-reversal process acts on its NRM.

The occurrence of self-reversal in igneous rocks has long been debated. Extensive investigations have been postponed because of the overwhelming evidence that reversely magnetised rocks generally were magnetised by the Earth's field in a reversed polarity state. However, a few examples of self-reversal of the NRM in volcanic rocks have been reported: the Haruna dacite in Japan¹⁷, certain oceanic basalts^{18,19} and a basalt from Germany²⁰. Partial self-reversal leading only to a reduction of NRM intensity at room temperature, but not to a completely antiparallel alignment of the NRM direction with respect to the ambient geomagnetic field, was observed in an historical lava flow of Mount Etna²¹. Furthermore it has been shown in continental basalts^{22–24} that thermoremanent magnetisation (TRM) produced during moderate heat treatment in the laboratory can acquire self-reversal or at least partial self-reversal characteristics at room temperature.

Self-reversal processes can be classified into three categories according to different physical mechanisms: (1) A one-constituent model in which the direction of spontaneous magnetisation changes sign at a certain temperature (Néel's²⁵ P- or N-type ferrimagnets). (2) A two-constituent model in which negative exchange interaction across the boundary of two magnetic phases plays the essential role. (3) A two-constituent model in which magnetostatic interaction between two phases leads to complete or partial self-reversal of remanence.

I have sampled the reversed basalts of Laschamp (site F³) and Olby (site B³), and one normally magnetised flow to the west of Puy de Barme. The NRM directions obtained after alternating field cleaning are similar to those observed by Bonhommet²⁶, but also some normal samples have been found in the reversed

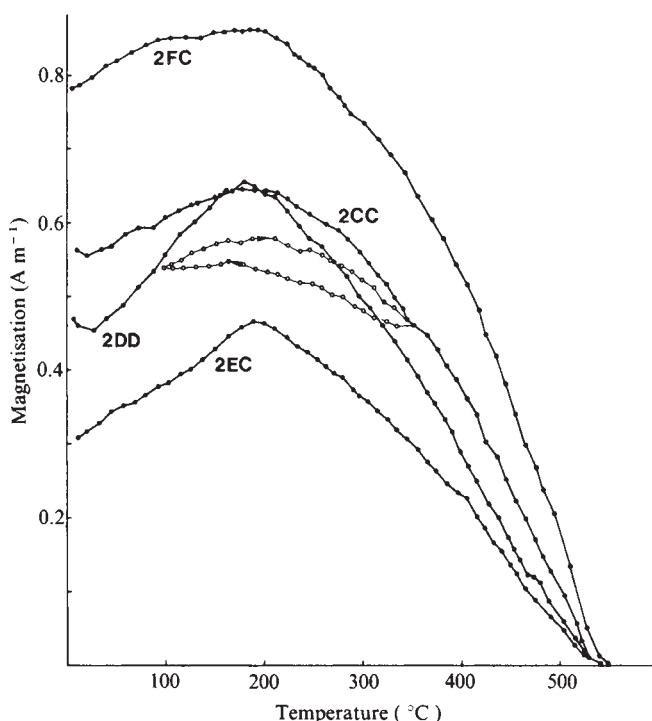


Fig. 1 Partial self-reversal of natural remanent magnetisation during heat treatment in four basalt samples collected from the reversed Olby flow. Heating rate, $10^{\circ}\text{C min}^{-1}$.

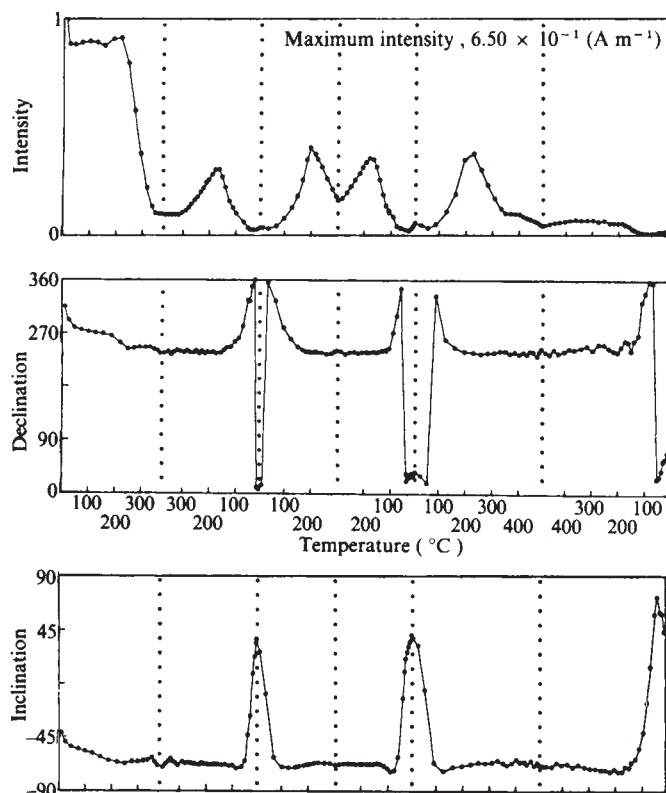


Fig. 2 Complete self-reversal of natural remanent magnetisation of an Olby basalt sample (QB 2 BC) during thermal treatment. Inclination, declination and intensity have been plotted separately against temperature. Three heating and cooling cycles follow each other. Note, the sharp peaks of the intensity curve around 200 °C are caused by the self-reversal process which leads to a normal remanence direction below 50 °C in all three cooling cycles. Above 200 °C the remanence direction is reversed and antiparallel to the room temperature direction.

Olby flow. Rockmagnetic studies by Whitney *et al.*²⁷ reported that both reversed lavas are highly oxidised without any indication of a self-reversal of NRM. My observations are that the rock-magnetic properties and other physical parameters (for example, oxidation state, porosity) of the lavas investigated vary to a large extent from flow to flow as well as within the flows.

The magnetic properties of the reversely magnetised Olby flow are particularly interesting and show that the NRM of at least this flow has self-reversal characteristics. Several samples of the flow have been subjected to continuous thermal demagnetisation²⁸. Figure 1 shows the NRM intensity behaviour during thermal treatment of four Olby samples which, before this experiment, were a.f.-cleaned in fields of up to 10 mT to remove secondary soft components. Up to 200 °C all samples show a peculiar increase in intensity amounting to up to 50% of the initial value. No directional NRM changes are observed during heating up to 550 °C. Sample 2CC shows that the demagnetisation curve is repeatable between 100 and 350 °C when the temperature is lowered and raised again. Therefore the initial intensity increase can be explained only by a partial self-reversal process acting in these samples. Below 200 °C an apparently normal NRM component is coupled to the pre-dominant reversed magnetisation the intensity of which is reduced in this way at room temperature.

Using the same demagnetisation technique as in Fig. 1, a complete self-reversal of NRM has been found in another Olby flow sample (Fig. 2). During the first heating a soft, viscous magnetisation component of normal polarity is removed up to 200 °C. This component obscures the behaviour of the stable NRM components in this temperature range. Further heating to 400 °C reduces the intensity, but leaves a stable reversed NRM direction which has a southwesterly declination and steeply

negative inclination as found often in the Olby flows²⁶. When cooling from 400 °C to room temperature in zero magnetic field ($H < 20$ nT) at first an intensity increase is observed till 170 °C which is followed by a sharp decrease in NRM intensity. At 150 °C the declination component begins to change direction. At about 40 °C the magnetisation changes sign and a normal NRM is built up roughly antiparallel to the former NRM direction. The same features are recognised in the subsequent second and third heating and cooling cycle (Fig. 2), where, during heat treatment, the NRM changes sign at temperatures between 50 and 70 °C. Thus, although their magnetisation behaviour is different, all three cycles lead to a complete self-reversal in this Olby flow sample.

At the present stage of investigation a unique answer to the self-reversal process itself cannot be given: one-phase or two-phase models may apply. On the basis of ore microscopic observation and thermal demagnetisation characteristics of NRM a two-constituent model is currently preferred. In contrast to earlier findings²⁷ I have optically identified only low temperature oxidised titanomagnetite in these Olby flow samples. Therefore the thermomagnetic curves may be controlled by two magnetic phases with different Curie temperatures (T_c): a titanomagnetite with $T_c \approx 180$ °C and a titanomaghemite with $T_c \approx 350$ °C (see Fig. 2). The sharp drop in NRM intensity during heating and cooling around 200 °C is caused by the reversal process, but seems to be incompatible with P-type magnetisation curves because of its peaked appearance. The disappearance of the sharp magnetisation peak at 200 °C during cooling in the third cycle may indicate that the Ti-maghemite has been destroyed by heating to near 500 °C. Thus a negative magnetic interaction between two phases may cause complete or partial self-reversal of NRM in experimental conditions. It still has to be shown that such an interaction took place during the original NRM formation. However, if the titanomagnetite formed at temperatures below 180 °C by low temperature oxidation, such a process would lead to a reversed NRM direction in the Olby flow in the presence of a normal geomagnetic field direction.

The reversely magnetised Laschamp flow carries highly oxidised titanomagnetite and complete self-reversal of NRM during laboratory experiments has not yet been proved. However, this lack does not dismiss the possibility of self-reversal for this flow, in particular as recent investigations²⁹ have demonstrated that self-reversal may occur in strongly oxidised titanomagnetite, but may not be reproducible by laboratory experiment.

The present experiments recall strongly the need for thorough rock-magnetic investigations³⁰ when ascribing reversed magnetisation directions in igneous rocks to reversals of the geomagnetic field.

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Palaeomagnetic constraints on Greater India's underthrusting of the Tibetan Plateau

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A minimum estimate of the magnitude of Greater India's underthrusting along the Main Central Thrust beneath its former leading edge and beneath the Tibetan Plateau to the north is reported here. A comparison of newly obtained late Palaeozoic and Mesozoic palaeomagnetic results from the Tibetan Sedimentary Series (Thakkhola Graben, north central Nepal) with results from the Indian subcontinent indicates an excess anticlockwise rotational movement of Greater India with respect to its former leading edge of 10–15°. Assuming that the pivot point for this differential rotation is located to the west of the Western Himalayan Syntaxis, this magnitude corresponds to a minimum underthrusting of continental lithosphere of 200–350 km at the longitude of central Nepal.

Large scale underthrusting of Greater India beneath the Tibetan Plateau has been postulated repeatedly on geological^{1–3} and geophysical grounds^{3,4} to explain the thickness of the crust beneath the Tibetan Plateau, about twice as thick as the average continental crust. This postulate conflicts with the established notion that buoyancy constraints^{5,6} preclude large scale subduction of continental lithosphere. Recent studies⁷ suggest, however, that such subduction over several hundred kilometres may be possible within the range of plausible values for model parameters.

The magnitude of Greater India's underthrusting beneath Asia can be constrained palaeomagnetically. Favourable conditions are: (1) northwards-directed underthrusting proceeded at first beneath Greater India's former leading edge which is preserved at the surface and can be studied; and (2) underthrusting resulted largely from an anticlockwise rotational

movement whose magnitude can be determined relatively easily. Besides, understanding of the evolution of the India–Asia collision has advanced considerably in recent times. Mutually supporting geological data^{3,8,9}, seafloor spreading analyses^{3,10,11}, and palaeomagnetic data^{12–14} indicate conclusively that welding of India to Asia along the Indus–Tsangpo suture zone (Fig. 1) occurred from about the late Palaeocene until maybe the late Eocene. Continental subduction during this phase was probably only of a minor magnitude^{3,8}. After the intimate contact had been established, the Indus–Tsangpo suture zone became immobilised and the India–Asia convergence slowed down considerably. A new zone of weakness developed about 100–200 km further to the south within Greater India and this zone evolved into the Main Central Thrust. Renewed convergence of intracontinental character occurred along the Main Central Thrust since possibly the Oligocene^{9,11}. Greater India thus underthrust its former leading edge and beyond, beneath the Tibetan Plateau. Powell and Conaghan^{3,4} postulated initially that Greater India's underthrusting may have proceeded beneath the full width of the Tibetan Plateau (over ~1,500 km at the longitude of far eastern Nepal). Powell¹¹, however, has recently reduced this estimate considerably.

Seafloor spreading analyses^{10,11,15–17} show that post-collisional convergence of India with Asia was characterised by an appreciable anticlockwise rotational movement of the Indian plate with respect to Asia. This can be interpreted as the main cause for the development of the pronounced asymmetrical pattern plan of the Himalaya^{11,18,19}. Our estimate for the magnitude of underthrusting along the Main Central Thrust is based on a comparison of newly obtained palaeomagnetic results from the former leading edge of Greater India with palaeomagnetic results from the Indian subcontinent (summarised in ref. 14). This method is particularly suited for the determination of the rotational component of underthrusting, but is much less precise in the determination of a possible northwards translational component of underthrusting.

Palaeomagnetic results representative of Greater India's former leading edge were obtained from late Palaeozoic and Mesozoic carbonates and sandstones from the Tibetan Sedimentary Series of the Thakkhola Graben, North Central Nepal. This region was chosen because of its relatively easy access, its detailed geological mapping^{20–22}, and the much lower degree of metamorphism than is prevalent in adjacent regions of the Tibetan Himalaya. Moreover, the Main Central Thrust is parti-

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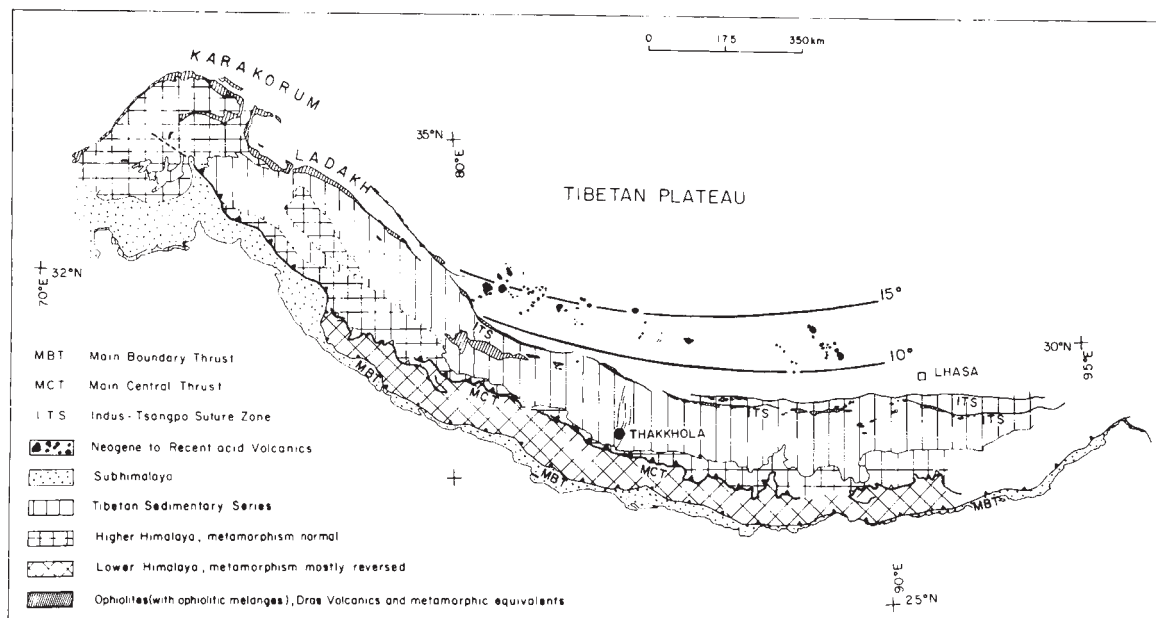


Fig. 1 Main structural features of the Himalaya after Gansser⁸. The dashed line indicates the westwards extension in the subsurface of the Main Boundary Thrust/Main Central Thrust complex according to seismic observations^{30,31}.

Table 1 Summary of Palaeomagnetic directions

Rock unit*	Age	N†	Dec(°)	Corrected for bedding Inc(°)	k	$\alpha_{95}(^{\circ})$	Fig. 2a Ref.
Secondary 'collision' component (50–60 Myr)							
Thinigaon Lst.		17	6.5	–9.5	39	6	1
Kagbeni Sst.		22	2	–12	16	8	2
Dzong Sst.		67	348	–6.5	23.5	3.5	3
Kagbeni Sst.		18	347.5	–5	16.5	8.5	4
Jomosom Lst.		77	346	–4	35	2.5	5
Jomosom Qzt.		36	346	–16	32	4.5	6
Primary component							
Dzong Sst.	Lower Aptian	78 (95)	326	–61.5	12	5	7
Kagbeni Sst.	Wealden	34 (40)	333.5	–56.5	15.5	6.5	8
Kagbeni Sst.	Wealden	24 (34)	322.5	–55.5	14	8	9
Lumachelle Fm.	Dogger	27 (43)	308	–61	12	8.5	10
Jomosom Lst.	Lias	22 (55)	308	–59.5	10.5	10	11
Jomosom Lst.	Lias	45 (82)	328.5	–42	14	6	12
Jomosom Qzt.	Rhaetian	32 (55)	323	–48	11	8	13
Thinigaon Lst.	Norian	22 (76)	324.5	–43	10	10	14
Thinigaon Lst.	Ladinian–Carnian	35 (57)	329	–47	21	5.5	15
Thini Chu Fm.	M?Permian	41 (40)	291.5	–63.5	10	7.5	16

Mean directions and statistical parameters were obtained according to ref. 38.

*Formation names and ages are according to refs 22, 28 and according to ref. 37 for the sampled part of the Thini Chu Fm.

†The number of specimens wherein the component concerned could be identified. The number of samples collected are in parentheses.

cularly well developed, and has been studied thoroughly²² in the region south of the sampled location.

A summary of significant palaeomagnetic data is given in Table 1; a more detailed account of results will be presented elsewhere (our work, in preparation). Altogether more than 750 core samples were drilled from cliff sections bordering the Kali Gandaki river in the tract between Shang (28°45'N 83°44'E) in the south and Kagbeni (28°49'N 83°46.5'E) in the north, and also in cliffs immediately north of the Muktinath sanctuary (28°48'N 83°50'E). We restricted our sampling mainly to the Mesozoic and to a lesser extent the late Palaeozoic carbonates and sandstones. These are very well exposed in a WNW–ESE trending belt situated ~10–20 km north of the main range, formed by the impressive Dhaulagiri and Nilgiri–Annapurna massifs. Middle and early Palaeozoic carbonates exposed immediately south of the sampled region, that is, in the north-face of Nilgiri, show a southwards increasing metamorphism

associated with formation of the Main Central Thrust and have not been sampled for that reason.

In general two specimens per sample were thermally demagnetised, using furnaces as described by McElhinny *et al.*²³, in about 10 steps over a temperature interval determined on the basis of more elaborate pilot demagnetisation studies. The directional content of the specimens was analysed from Zijderveld plots²⁴. Directions of characteristic components were determined using a principal component analysis program adapted from Kirschvink²⁵. Simultaneous breakdown of a multicomponent system of directions was fairly commonly observed in the carbonates. Whenever such specimens failed to respond satisfactorily to the Zijderveld-type analysis we analysed them subsequently for cryptic components with non-unique blocking temperature spectra²⁶, following Kirschvink's²⁵ method. Unit weight was given to specimen directions throughout the statistical analysis.

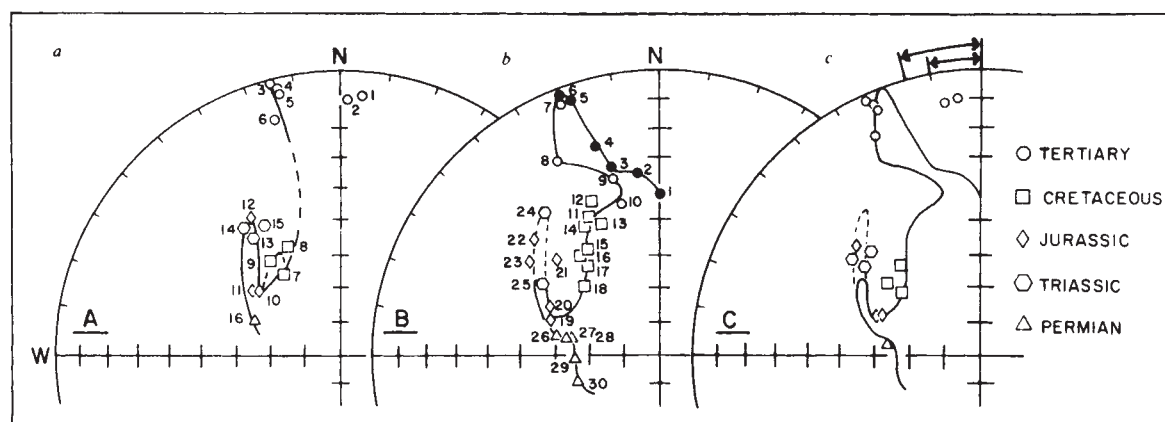


Fig. 2 Comparison of palaeomagnetic directions observed for the Thakkhola region (a) with directions expected for this region (b) according to palaeomagnetic data from the Indian subcontinent^{13,14} assuming that no relative movement between the two regions has occurred. The observed and the expected directions agree very well after correction for a 10° ('collision' component, 1–6) to a 15° (primary component, 7–16) differential rotation between the two regions (c). a, Secondary 'collision' component (1–6; 50–60 Myr) and primary components (7–16; Lower Aptian to Middle? Permian) as detailed in Table 1. b 1, present field direction; 2, 5–22.5 Myr (DSDP); 3, 25–35 Myr (DSDP); 4, 35–45 Myr (DSDP); 5, 45–55 Myr (DSDP); 6, Sanjawi Lst. (Palaeocene–Eocene); 7 Upper Brewery Lst. (base Upper Palaeocene); 8, 55–65 Myr. (DSDP); 9, Upper Normal Deccan Traps (60–65 Myr.); 10, Lower Reversed Deccan Traps (60–65 Myr.); 11, 12, Tirupati beds (Lower Cretaceous); 13, 65–75 Myr. (DSDP); 14, Satyavedu beds (Lower Cretaceous); 15, Sylhet Traps (Lower Rajmahal Traps); 16, 17, Goru Formation–Parh Lst. (Aptian–Albian to Santonian–Campanian); 18, Rajmahal Traps (100–105 Myr.); 19, Loralai Lst. (Middle–Upper Jurassic); 20, Chiltan Lst. (Upper Jurassic); 21, Mean Middle Jurassic pole for Australia transferred to the Indian plate; 22, as above, mean Lower Jurassic pole; 23, As above for the mean Middle Jurassic pole of Antarctica; 24, Parsora beds (Upper Triassic); 25, Pachmarhi beds (Upper Triassic); 26, Kamthi beds, Wardha Valley (Permo–Triassic, 14); 28, Panchet beds (as above); 29, Kamthi beds, Wardha Valley (as above, 36); 30, Talchir beds (Permo–Carboniferous).

The specimens generally showed a fairly complex magnetic pattern. A recent field component of normal and to a lesser extent reversed polarity predominated, together with another secondary component and a primary component which were both mainly of normal polarity. Comparison with the Indian Tertiary apparent polar wander path (APWP)²⁷ shows that this secondary component stabilised between 50 and 60 Myr ago. This is also the time interval for which Krummenacher²² observed a notable resetting of K–Ar ages in central and eastern Nepal. Both the palaeomagnetic and the K–Ar data thus clearly reflect the initial collision of Greater India with southern Asia. Apart from the three magnetic components described above we also observed a west- to westnorthwestwards-directed secondary component of low downwards inclination and mainly of normal polarity. Its origin may be related to a similarly aligned pattern of *b*-axes^{22,28,29} which is well developed in the Tibetan Sedimentary Series. Secondary components of both normal and reversed polarity which originated during formation of the Main Central Thrust were dominantly present only in Carboniferous–Devonian rocks taken from the most southern sampling locality.

In the absence of contrary evidence we interpret the mean directions for the primary and secondary 'collision' components observed in the Tibetan Sedimentary Series of the Thakkhola Graben to be representative for Greater India's former leading edge, notwithstanding possible local distortions in its western extremity¹⁴. Figure 2 compares these mean directions (*a*) with the directional pattern expected for the Thakkhola region according to the Indian APWP (*b*). The observed and expected directional patterns agree very well after correction for an excess anticlockwise rotational underthrusting over 10° ('collision' component) to 15° (primary component) of Greater India with respect to its former leading edge (Fig. 2c). No clear indication for a northwards-directed translational underthrusting of Greater India beneath the Tibetan Plateau along the Main Central Thrust, or maybe along the Main Boundary Thrust⁹, was detectable in our palaeomagnetic results because of the inherent poor angular precision of such a determination. The asymmetrical pattern in the plan of the Himalaya^{17,18,19} indicates that the pivot point of the rotational underthrusting has to be looked for in the northwestern part of the Indian subcontinent. As a first approximation we assume its location at the western extreme of the Indus-Kohistan Seismic Zone^{30,31}. This zone represents the subsurface extension to the west of the Western Himalayan Syntaxis of the Main Boundary Thrust/Main Central Thrust complex, which thrusts, for all practical purposes, are indistinguishable from each other in the region immediately to the east of the Syntaxis. If we assume for the central and eastern part of the Himalaya that this differential rotation was restricted to rotational underthrusting along the Main Central Thrust alone, then its observed 10–15° magnitude corresponds at the longitude of central Nepal to a minimal underthrusting of 200–350 km of Greater India beneath its former leading edge and further north beneath the Tibetan Plateau.

As summarised by Molnar *et al.*¹⁹, geological, gravity and seismic data suggest an ~15° northwards dip of the Main Central Thrust. Assuming this dip to be uniform, the surface projection of the frontal part of underthrust Greater India can be located as shown in Fig. 1 according to rotational underthrusting values of 10° and 15°, respectively. Depths to the top of the frontal edge of this underthrust slab of continental lithosphere range from ~60 km in the west to ~120 km in the east taking the higher value of 15° of rotational underthrusting. It has been demonstrated³² that the calc-alkaline volcanism of island arcs is caused by melting of oceanic lithosphere at similar depths of 80–120 km. As Fig. 1 shows, there is a striking coincidence between the surface projection of the subducted slab of what in the present case happens to be continental lithosphere and a zone of Neogene to Recent calc-alkaline volcanism in southern Tibet. This zone is shown (Fig. 1) according to the 1:3,500,000 structural map of Gansser⁸, although other workers^{33–35} suggest a wider distribution of calc-alkaline volcanism. This coincidence supports our palaeomagnetic interpretation that continental

lithosphere can subduct in Himalayan-type conditions over distances of at least several hundreds of kilometres.

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A man-made hot spring on the ocean floor

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An instrument package emplaced in a deep-sea drill hole for the Glomar Challenger measured a rise in temperature at the bottom of the hole from 78°C shortly after drilling to 150°C after 42 days. The increase, not predicted by temperature measurements made before and during the drilling, is probably the result of hot water rising from below and flowing out of the hole into the ocean. We describe here this first recorded man-made hot spring on the ocean floor.

A downhole seismometer package was placed from the Glomar Challenger in DSDP hole 482-C (22°47.34' N, 107°59.57' W), located at the mouth of the Gulf of California about 12 km from the East Pacific Rise south of the Tamayo Fracture Zone¹. The hole was drilled in 3 km of water through 143 m of sediments and 47 m of massive basalt. The instrument (Fig. 1), constructed at the Hawaii Institute of Geophysics,

consists of a downhole sensor package containing thermal sensors, seismometers, tiltmeters, and associated electronics. Signals from the sensors are multiplexed and digitised into a 16-channel format for transmission by wire to a data recording and power package located on the ocean floor. The recording package is connected by floating rope to an anchor-float assembly that can be commanded to surface for data tape recovery and refurbishing of the system. A complete description of the experiment will be published elsewhere.

During the drilling, temperature was measured in the sediments with the Uyeda temperature probe, whose sensor is inserted into the sediments below the drill bit and is thus not affected by the drilling process. The temperatures show a linear trend (Fig. 2) compatible with the heat flow measurement of 12 HFU made during the site survey², if the conductivity of the sediments is assumed to be $2 \text{ mcal cm}^{-2} \text{ s}^{-1} \text{ }^{\circ}\text{C}^{-1}$. Using the same assumptions, the equilibrium temperature at the bottom of the hole should be between 90° and 110°C , depending on the conductivity of the basalt. As this value was below the design maximum of the instrument package (130°C), the sensor package was put in position on 3 February 1979.

During emplacement of the downhole seismometer package, temperature measurements were made during two time periods separated by 11 h. The first period was 30 min long and started about 7.5 h after the last water was pumped into the hole. The temperature at the end of this period was $20 \pm 2.5^{\circ}\text{C}$. By the end of the second period, 13.5 h later, the water temperature at the bottom of the hole had risen to $78 \pm 2.5^{\circ}\text{C}$, a rate of more than 4°C h^{-1} . This increase was assumed to be caused by the reheating of the hole after cooling by the drilling water.

After the second measurement period, the instrument was left to record data for 42 days and then recovered by the RV Kana Keoki on 17 March 1979. When the recording package was recovered, the sensor package was again monitored in real time. Although most of the electronics, including the multiplexer and the a to d converter were still operating, few of the sensors seemed to be working and there seemed to be little value in keeping the system operating. Therefore, the sensor package was removed from the hole. The package showed obvious signs of excessive heat; both tiltmeters had exploded, and

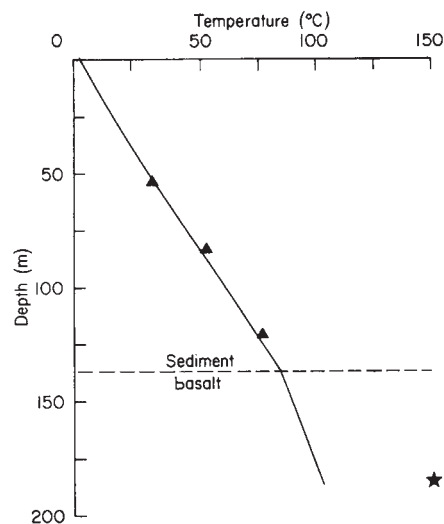


Fig. 2 Extrapolation of temperature measurements made while drilling (▲) yielded a temperature of about 100°C at the bottom of the hole. After 42 days, however, the temperature measured at the bottom (★) was about 150°C .

components and circuit boards that had been green before emplacement were now dark brown. Two components had mechanically shifted during emplacement, causing electrical short circuits through seawater to the recording package; however, the temperature sensors and circuitry were apparently undamaged. Although no data were obtained from the recording package, an additional 30 min of temperature information obtained during recovery showed that the temperature had risen dramatically during the time since emplacement. Because the temperature measurement obtained during recovery was greater than the maximum temperature for which the system had been calibrated (130°C), the sensor package was recalibrated at temperatures up to 155°C on its return to the Hawaii Institute of Geophysics. The new calibration values agree with those taken before the experiment up to 130°C ; the values above 130°C , while no longer linear (all components in the package were rated only to 125°C), were repeatable. Thus we believe that the $150 \pm 5^{\circ}\text{C}$ value obtained after 42 days in the hole is valid.

The discrepancy between predicted temperature at the bottom of the hole and the measured value can be explained if the hole acts as a conduit allowing hot water to rise from the more permeable basalt through the relatively impermeable sediments, forming a hot spring in the ocean bottom. The hole was probably heated over a period of time as hot water from below continued to enter the hole.

While there are other possible explanations for the temperature increase, we believe that the hot spring hypothesis is the most probable cause. Many authors have suggested that hydrothermal circulation is responsible for erratic heat flow values in the ocean³⁻⁹. Heat apparently is released in large amounts in regions where sediments are thin or where basement protrudes through the sediments⁵. In areas where sediments are thick, heat flow tends to be lower than expected because of the blanketing effect of the sediments. The theory of Parsons and Sclater³ predicts a value of 17 HFU for crust aged 0.5 Myr (hole 482-C) for conductive heat flow as compared with the measured value of 12 HFU, indicating that heat is being lost by means other than conduction even before the drilling. The drill hole is an efficient conduit whereby heat can flow through the low permeability sediments by convection. Recent discoveries of natural hot springs at ridge crests⁶ also document the importance of hydrothermal circulation in heat transfer near ridge crests and in the placement of hydrothermal ore deposits.

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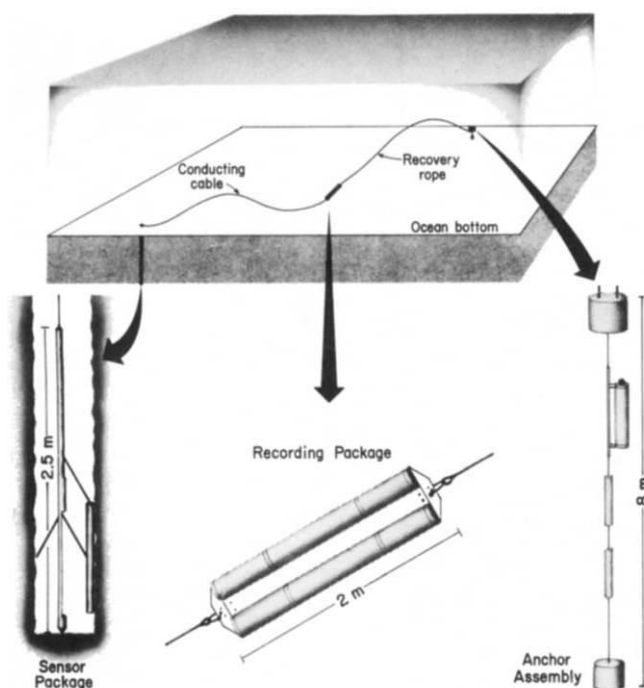


Fig. 1 The ocean sub-bottom seismometer. The sensor package was lowered to the bottom of the hole through the drill pipe by the Glomar Challenger. The pipe was then stripped from the wire and the recording package and anchor assembly attached and lowered to the ocean bottom.

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An Acheulean industry with *Elephas recki* fauna from Namib IV, South West Africa (Namibia)

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Namib IV is located in the linear dune sea of the central Namib Desert (Fig. 1), where different generations of fossil calcrete have been exposed by sand shift in an interdune flat. Mid-Pleistocene faunal remains (including *Elphas recki*) were scattered over 62,500 m² of the valley floor, some bearing residual traces of red calcrete. The industry may be closely paralleled from certain East African sites (Olorgesailie¹, Kilombe², PDK and HEB 1/2 and 3, Olduvai Bed IV (ref. 3)), suggesting a date between 400 and 700 kyr BP. I show here that Namib IV provides the first dated evidence for the presence of Acheulean man in South West Africa, the earliest Pleistocene faunal remains from the Namib Desert and a *terminus post quem* for the ecological change from savannah to sand desert.

Randomly orientated artefacts were scattered on the present ground surface, the presence of residual calcrete patches on the ventral surface of some artefacts and bones suggesting that they

had recently been weathered from a formerly extensive calcrete deposit of which a few remnants are still visible. Preliminary reconnaissance of the total distribution area was completed in 1978, and a statistical analysis made of 394 artefacts found within one randomly-selected grid square (22,500 m²), together with the collection of all faunal material from the site. The implement assemblage included bifaces (handaxes and cleavers) comprising 30% of the total, the rest being spheroids (2%), chopper/cores (7%), small flake tools (58%), working debris, débitage and unretouched flakes. Raw materials were very varied, principally yellow, pink and grey quartzite with lesser quantities of dolomite, dolomitic marble and diamictite obtained from cobbles in the canyon of the Kuiseb river, some 8 km north of the site. Some relationship between raw material and artefact type was evidenced, for example, by the dolomite spheroids (bolas) and the large cleavers which were almost exclusively made of quartzite. Smaller flake tools and scrapers were made from quartz, obtained from veins in the local outcrops of mica-schist.

There was a marked tendency for the bifaces to be made on flakes, a frequent method of manufacture being the splitting of a large pebble and the trimming of a few flakes from one side only, leaving the other consisting almost entirely of cortex. This was particularly evident on the cleavers, where as few blows as possible had been used consistent with producing a serviceable edge of the required shape. The handaxes, which tended to be pick-like or chisel-ended variants of the 'classic' pointed handaxe, composed only 5% of the whole industry. The cleavers, representing a further 25% of the industry, were larger and heavier with a marked tendency to be made on side-struck flakes. All bifaces showed traces of heavy usage.

The manufacture of bifaces from large flakes is characteristic of the Acheulean and it has been suggested⁴ that the development of the technique may be crucial in differentiating Acheulean industries from Oldowan. Typologically although Namib IV may closely be paralleled by East African industries from

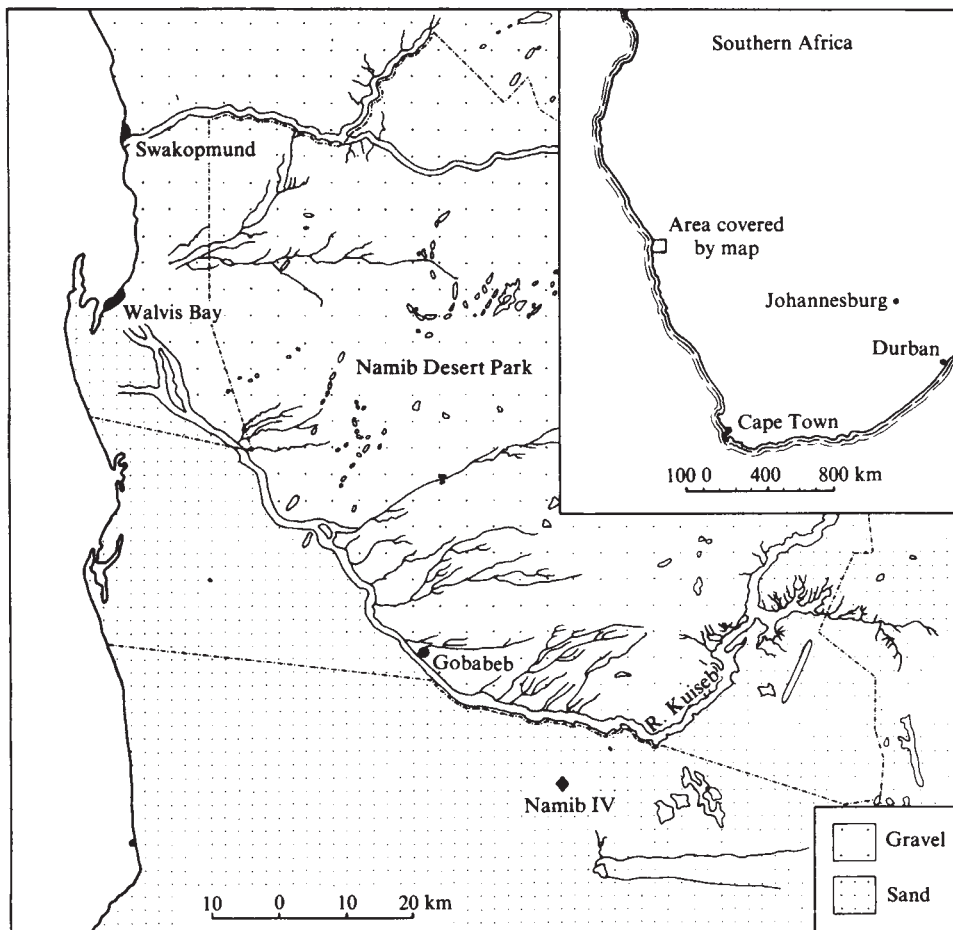


Fig. 1 Map of the central Namib Desert; inset shows its location in southern Africa.

Olorgesailie, Kilombe and Olduvai IV, the thickness of the bifaces, low number of flake scars and lack of secondary working suggest that it might be slightly earlier in date, although firmly placed within the mid-Pleistocene Acheulean technocomplex. Certain features of the Namib IV assemblage, including the dominance of cleavers over handaxes, are in sharp contrast to the East African sites, although the complete absence of picks and the presence of sub-rectangular cores, spheroids and large number of flake tools reinforces the general similarity. Preliminary readings from Kilombe⁵ suggest that the site antedates the Brunhes–Matuyama transition, and is therefore >700,000 yr old. Isaac¹ has stated that it is likely that Olorgesailie may date to before 400 kyr BP and it is suggested on typological grounds that Namib IV is of similar antiquity. The fauna included the mineralised remains of *Elphas recki*, together with one indeterminate alcelaphine antelope and one other medium-sized antelope. The *Elphas* remains fortunately included tooth fragments whose thin enamel, hypsodonty and tight enamel folding suggest that a late stage of *E. recki* is represented, probably comparable with Maglio's Stage 4 (ref. 6) as represented at Olduvai Bed IV, confirming the mid-Pleistocene date for the site.

The precise mode of formation of the calcrete deposit is as yet uncertain but it is undoubtedly connected with the former presence of surface water, probably a small ephemeral lake. The climatic shift which took place after the mid-Pleistocene resulted in an ecological change from savannah grassland with sufficient fodder for elephants to the present sand desert; a desiccation that resulted in a lowered water table and the formation of evaporites incorporating artefacts and bones lying on or near the former lake margins. The paucity of the bone refuse makes it impossible to say whether the artefact concentration represents a butchery site or a camp, but it is unlikely that hunters would have remained in the vicinity of their kills for more than a few days, due to competition from other, larger carnivores⁷. A butchery site visited on many occasions is the most likely hypothesis, an argument based on what Perkins and Daly⁸ called a 'schlepp effect', namely that bones which are bulky or of little nutritive value are unlikely to be removed from kill site to camp. Klein⁷ notes that the occurrence of elephant bones at Pleistocene base-camp sites in Africa is very rare, and that they are almost totally absent from the extensive faunas studied from South African cave sites. Acheulean butchery sites like Elandsfontein 'Cutting 10'⁷ are, conversely, often rich in elephant remains and sometimes, as in the case of Namib IV, these come from adult or sub-adult individuals.

Although the occurrence of Acheulean material in the Namib has been previously noted⁹ no associated fauna was preserved. It is hoped that an extension of the 1978 study of Namib IV will reveal typological and distribution information concerning the remains of the Acheulean in South West Africa, together with further details of the Pleistocene environment.

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***In vitro* flowering of embryoids derived from mature root callus of ginseng (*Panax ginseng*)**

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Seedlings of various crops pass through a juvenile phase during which they cannot be induced to flower¹. The delay in flowering caused by a long juvenile phase may last for years and is a major problem in breeding such crops. In field conditions, ginseng (*Panax ginseng* C. A. Meyer) usually flowers in the third year after germination², and breeding programmes have been hindered by the long time needed for genetic analysis. We report here a repeatable precocious flowering of embryoids derived from mature ginseng root callus cultured in a chemically defined medium. These embryoids produce flowers and fertile pollen without establishing normal seedlings.

In vitro somatic embryogenesis of ginseng was first reported by Butenko and her colleagues³, who found that spontaneous somatic embryogenesis occurred on cultured callus derived from various tissues, including leaf, petiole, anthophore and stem. However, attempts to regenerate whole ginseng plants from these embryoids failed. We have since reported conditions for such regeneration⁴.

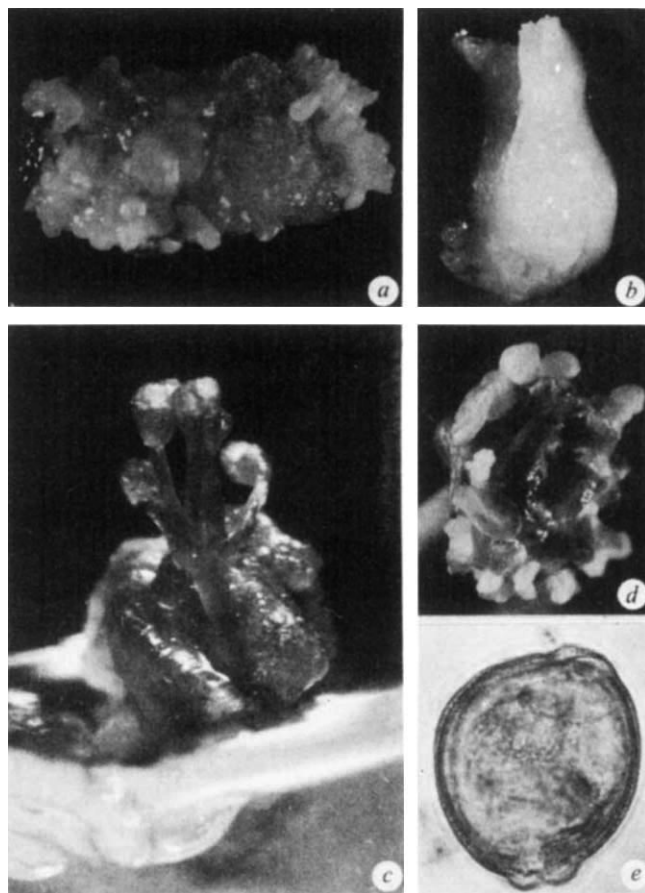


Fig. 1 a, Cluster of embryoids derived from mature root callus ($\times 5$); b, a dicotyledonary embryoid ($\times 40$); c, flowers developed from an isolated embryoid ($\times 4$); d, a minuscule flower with well-developed anthers ($\times 10$); e, fertile pollen of well-developed flowers ($\times 500$).

Callus was obtained by aseptically culturing pith tissue of a mature ginseng root on an agar medium consisting of 2% sucrose, vitamins and the major and minor salts of Murashige and Skoog⁵. This basal medium (BM) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, 5 mg l⁻¹) kinetin (2 mg l⁻¹). The cultures were kept in a growth chamber at 26±1 °C in darkness. When subcultured, the callus grew most rapidly in BM plus 2,4-D (2 mg l⁻¹), kinetin (1 mg l⁻¹) N⁶-(Δ²-isopentenyl)-adenine (2iP, 1 mg l⁻¹). Embryoids were obtained by reducing the 2,4-D to 1 mg l⁻¹ and omitting kinetin and 2iP (Fig. 1a, b). They failed to develop and remained 2–3 mm long. Some of them, however, gave rise to a mass of friable callus, which produced more embryoids when subcultured on BM plus gibberellic acid (GA), adenine sulphate, tyrosine and charcoal; the optimal combination of these factors was 0.1 mg l⁻¹, 40 mg l⁻¹, 50 mg l⁻¹ and 0.5% respectively. Such embryoids, however, failed to develop further in subculture.

To obtain further development, embryoids 2–3 mm long were isolated from the callus and subcultured either on half-strength BM supplemented with benzyladenine (BA, 1 mg l⁻¹) and GA (0.5–1 mg l⁻¹) or B5 medium⁶ supplemented with BA (1 mg l⁻¹) and GA⁴ (1 mg l⁻¹). Well-structured flowers were repeatedly observed when the embryoids were cultured on the supplemented B5 medium at 26±1 °C under a 16:8 light-dark regime, 1,500 lux (Fig. 1c). Two cotyledons of the cultured embryoid developed into a mass of green unorganised tissue, and the apical meristem located between two cotyledons transformed

into a peduncle terminated by a simple umbel with 3 to 15 flowers. These flowers were small, about 2–3 mm across. Each flower consisted of five-toothed green calyx, five yellowish green entire petals, 5 to 10 short stamens with oblong anthers, and two green pistils. These well-structured floral parts were minuscule but easily recognisable (Fig. 1d). About 90% of the pollen grains (Fig. 1e) were fertile as revealed by smearing and differential staining by Alexander's procedure⁷.

Thus embryoids have the potential to produce flowers directly in defined conditions. Compared with the intact plant systems used in studies of flowering, these embryoids have the advantage that many of them can be obtained in defined sterilised culture⁴, and within 1 month of subculture, as described here, flowers can be obtained. Thus the embryoid system could provide experimental material for studies of flowering without the need for the normal ginseng juvenile phase (3 yr).

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Luteinising hormone-releasing and anti-fertility properties of a glucagon-selective somatostatin analogue

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The hypothalamic tetradecapeptide, somatostatin (SRIF), inhibits the secretion of growth hormone (GH)¹ and numerous other hormones², including insulin and glucagon^{3,4}. Attempts to use SRIF as an adjunct in the treatment of diabetes mellitus^{5,6} met with limited success due to its short biological half-life^{7,8} and the undesirable diabetogenic activity of its insulin-lowering properties^{3,9,10}. Efforts at synthesis have yielded SRIF derivatives with prolonged GH-lowering activity which did not suppress glucagon or had equivalent insulin-inhibiting activity^{11–14} as well as several short-acting compounds with the appropriate glucagon specificity^{15,16}. A dodecapeptide analogue [des-Ala¹, Gly²] His^{4,5}-D-Trp⁸-SRIF (Wy-41, 747) has been identified that combines selective inhibition of GH and glucagon release with prolonged activity^{17,18}. However, in routine pharmacological tests chronic treatment of mature rats with Wy-41, 747 produced anti-reproductive effects resembling those described for luteinising hormone (LH)-releasing hormone (RH) and its agonists^{19–25}. We report here that Wy-41, 747, unlike SRIF and other of its analogues tested, releases LH, induces ovulation and inhibits pregnancy when administered before or after implantation; these properties are traditionally associated with the separate LH-releasing class of peptides.

Wy-41, 747 was administered to ten adult male and ten adult female (initiated on metoestrus of 4-d cycle) rats at a total dose of 900 µg per kg, given subcutaneously in three equally divided injections at 0900 h, 1500 h and at 0900 h on the following day.

A further group of ten males received SRIF in a total dose of 4.3 mg per kg, in view of its lesser potency and shorter half-life, delivered as six divided doses at 0900 h, 1000 h, 1500 h, 1600 h and 0900 h and 1000 h on the following day. Animals injected with vehicle (0.9% saline adjusted to pH 4.0 with HCl) served as controls. One hour after the final injection, serum was collected for radioimmunoassay of reproductive hormones^{21,26}. Wy-41, 747 caused a 2.9-fold increase in LH in males (control, 70±15 ng ml⁻¹; Wy-41, 747, 203±27 ng ml⁻¹) and a 21.9-fold increase in females (control 21±4 ng ml⁻¹; Wy-41, 747, 466±80 ng ml⁻¹). SRIF released no LH in males. Serum prolactin levels were reduced, although not significantly, by Wy-41, 747 in both sexes. The analogue depressed testosterone significantly in males ($P < 0.05$) but had no effect on oestradiol and progesterone in females. Thus Wy-41, 747, unlike its parent molecule SRIF^{2,27,28}, can release LH effectively and may also inhibit the release of prolactin as well as gonadal steroidogenesis.

Confirmation of the LH-releasing properties of Wy-41, 747 was provided by its ability to induce ovulation. At 1330 h on the day of proestrus, pentobarbital (50 mg per kg) was injected intraperitoneally to inhibit the spontaneous surge of LH necessary for ovulation. Test compounds were administered subcutaneously in saline, pH 4.0, between 1340 h and 1350 h and LH-releasing activity was determined by the presence of oviducal eggs the next morning (oestrus). LHRH was included as a standard by which to compare Wy-41, 747 and SRIF. As Fig. 1 shows, Wy-41, 747, unlike SRIF, induced ovulation in a dose-related fashion. Although very effective in this test, Wy-41, 747 was only 1/10 as potent as the endogenous LH-releasing peptide, LHRH. Preliminary evaluations of the ovulation-inducing activity for several other SRIF analogues indicate that the LH-releasing property is unique to Wy-41, 747.

A further group of pentobarbital-blocked proestrous rats was injected with the ED₁₀₀ dose of Wy-41, 747 necessary for ovulation (100 µg) and blood samples were taken by heart puncture 1 and 2 h later. Within 1 h the LH of the seven analogue-treated animals had increased to a level 6.6 times as great as that in three rats receiving saline; after 2 h the difference was 25.8 times. Wy-41, 747 also inhibited serum prolactin at both intervals after injection (1 h: saline control, 80±37 ng ml⁻¹; Wy-41, 747, 19±8 ng ml⁻¹; 2 h: saline control, 82±32 ng ml⁻¹; Wy-41, 747, 11±5 ng ml⁻¹).

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LHRH and its agonistic analogues, in spite of their fertility-enhancing classification (LH release, ovulation induction), consistently produce anti-fertility effects, including that of inhibiting pregnancy^{19,20}. Because this is presumed to be mediated by inappropriately increased endogenous LH levels^{21,22}, Wy-41,747, which possesses the LH-releasing property, was tested for its effect on pregnancy in rats. Beginning on day 1 of pregnancy (appearance of vaginal sperm) and continuing to day 7 (pre-implantation period), or beginning on day 7 and continuing to day 12 (post-implantation period), all compounds except SRIF were administered in a divided daily dose at 0900 h and at 1500 h. SRIF, because of its limited duration of activity, was administered in four divided daily doses at 0900 h, 1000 h, 1500 h and 1600 h. Autopsy and assessment of pregnancy status (the presence of at least one normal fetus was the criterion for pregnancy) occurred on day 14 (pre-implantation groups) or day 18 (post-implantation groups). Figure 2 shows that Wy-41,747 can terminate pregnancy when administered during either days 1–7 or days 7–12; in both tests it was approximately one-half as potent as LHRH. SRIF had little (pre-implantation) or no (post-implantation) inhibitory effect at high doses.

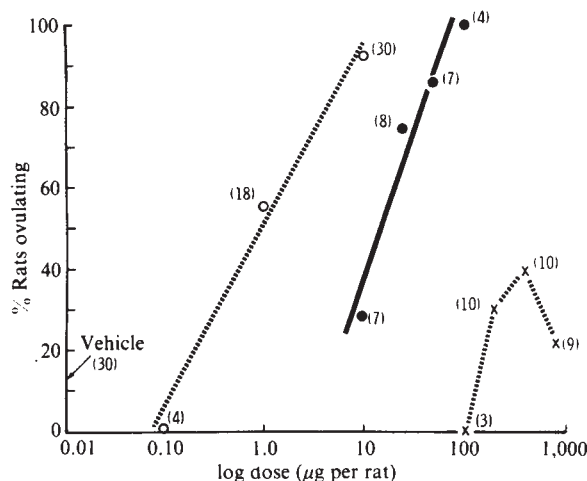


Fig. 1 Effect of LHRH (○), Wy-41,747 (●), and SRIF (×) on the induction of ovulation in pentobarbital-blocked proestrous rats. Numbers of rats are given in parentheses.

These results demonstrate that Wy-41,747, in contrast to SRIF and certain other SRIF analogues, has a reproductive pharmacological profile remarkably similar to that of LHRH. This SRIF derivative can release LH, induce ovulation and inhibit pregnancy before and after implantation, all integral properties of LHRH and LHRH agonists. Thus, the structural modifications of SRIF resulting in Wy-41,747 have resulted in a unique molecule with the high potency and the long-acting selectivity of the parent molecule (that is, ability to lower GH and glucagon) combined with characteristics of a separate class of peptides.

The anti-fertility effects of chronic administration of Wy-41,747, in addition to inhibition of pregnancy, include: in the male, decreased testicular and sex accessory weights, histological evidence of disorganised seminiferous tubules, inhibition of spermatogenesis; and, in the female, reduced folliculogenesis and uterine atrophy. These effects are strikingly similar to those reported for LHRH and its agonistic analogues^{21–25}. It has been proposed that the anti-reproductive action of LHRH/LHRH agonists occurs, in part, through an excessive release of LH, thereby reducing gonadal LH receptors (down-regulation) which is probably exacerbated by an associated decline in serum

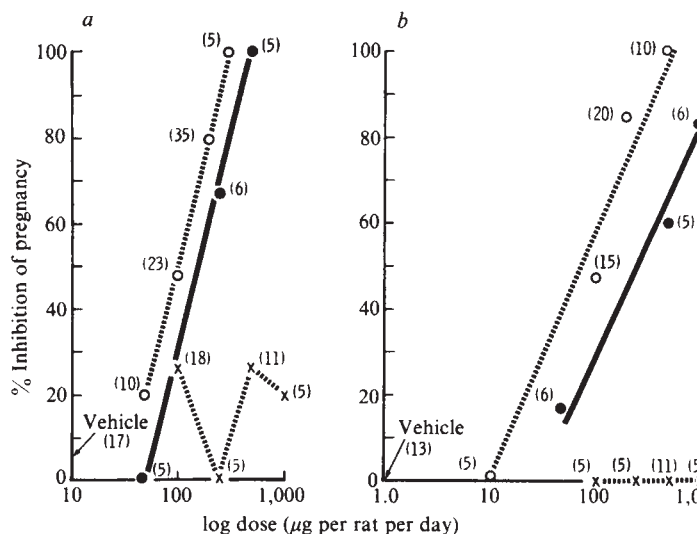


Fig. 2 Post-coital effect of LHRH (○), Wy-41,747 (●), and SRIF (×) in the rat. *a*, Inhibition of pregnancy as determined on day 14 after treatment during the days 1–7, pre-implantation; effect was characterised by lack of uterine implantation sites or resorbing sites. *b*, Inhibition of pregnancy as determined on day 18 after treatment during days 7–12, post-implantation; effect was characterised by resorbing sites and/or placental scars. Numbers of rats are given in parentheses.

prolactin levels^{21,22}. This, in turn, results in depressed gonadal steroidogenesis and a disruption of gonadal steroid-dependent tissues. The ability of Wy-41,747 to release LH and lower prolactin suggests that its anti-reproductive activity may occur through a similar pituitary-mediated downregulatory mechanism, although, as recently identified for LHRH and an LHRH agonist^{29,30}, direct gonadal effects may be involved as well.

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Proposed mechanism of cholinergic action in smooth muscle

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An increased turnover of phosphatidate and phosphatidyl inositol has been found in many tissues where hormones or neurotransmitters are postulated to raise Ca^{2+} influx¹, for example in smooth muscle²⁻⁴. However, the relationship between changes in phospholipid metabolism and changes in Ca^{2+} permeability was unknown. Following recent reports on the interactions of Ca^{2+} with phosphatidic acid in membranes and artificial systems, we investigated the hypothesis that phosphatidate accumulation mediates the action of cholinergic and other stimuli on Ca^{2+} influx. We report here that synthesis and accumulation of phosphatidate was accelerated in smooth muscle cells stimulated by carbamylcholine with a similar time course to that of contraction. This alteration in phosphatidate metabolism does not seem to result from an increase in intracellular Ca^{2+} or depolarisation of the cell membrane. Furthermore, submicromolar concentrations of phosphatidate rapidly produce contractions of isolated smooth muscle cells. These results support the contention that cholinergic-induced changes in membrane Ca^{2+} permeability in smooth muscle could be mediated by phosphatidate accumulation.

Several predictions of this hypothesis were evaluated. First, cholinergic stimulation should increase phosphatidate accumulation. Second, phosphatidate accumulation should occur independently of, and at least as rapidly as, the change in Ca^{2+} movement. Finally, exogenous phosphatidate should produce effects similar to those elicited by cholinergic stimulation. The use of enzymatically disaggregated smooth muscle cells from the stomach muscle of *Bufo marinus*⁵ afforded exceptional advantages in testing these predictions. The muscarinic response of those cells has been well studied⁶. Furthermore, changes in Ca^{2+} movements can be inferred from changes in the contractile state of the isolated cell, and their use obviates interpretive complications inherent to studies with intact tissues: heterogeneous cell populations, multiple diffusion barriers, and intercellular coupling⁷.

Changes in phosphatidate accumulation were assessed by following the incorporation of ^{32}P into the phospholipid. Within 10 s (a time when contraction is not yet maximal) after exposure to carbamylcholine, ^{32}P incorporation into phosphatidate was increased nearly threefold (Fig. 1). Similarly, brief periods of exposure to carbamylcholine increased phosphatidate content (assayed as glycerol-1-phosphate after saponification) from 340 to 570 pmol per aliquot cell suspension ($\sim 10^6$ cells). These data demonstrate that cholinergic stimulation increases phosphatidate accumulation, and this accumulation occurs rapidly enough to mediate changes in Ca^{2+} movement.

To assess whether the increased ^{32}P -phosphatidate labelling was independent of the increases in intracellular Ca^{2+} and/or the membrane depolarisation produced by cholinergic stimulation, the effects of K^+ on phosphatidate were studied. Exposure to high concentrations of K^+ produces contraction⁶ and would also be expected to depolarise the membrane. However, as K^+ failed to affect ^{32}P incorporation into phosphatidate, the effect of carbamylcholine on phosphatidate seems to be independent of membrane depolarisation and increased Ca^{2+} levels.

These studies are consistent with the hypothesis that increased phosphatidate accumulation may mediate the

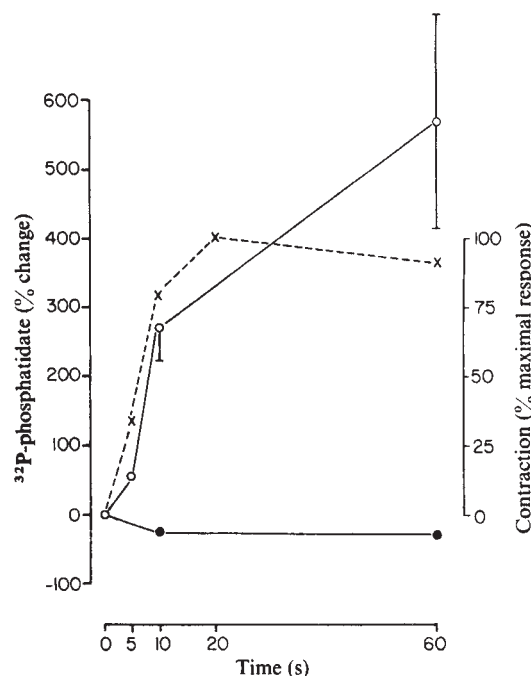


Fig. 1 Time course for the effect of carbamylcholine (carbachol) and KCl on ^{32}P -phosphatidate incorporation into phosphatidate in isolated smooth muscle cells. For comparison the time course of carbachol-induced contraction is shown (data replotted from Fay and Singer⁶). Suspensions of isolated smooth muscle cells were incubated for 15 to 30 min in amphibian Krebs-Ringer bicarbonate buffer containing ^{32}P -phosphatidate (10^{-4} to 10^{-5} M) before exposure to carbachol (10^{-4} M) or KCl (40 mM) for the times indicated. The cells were disrupted by sonication (Branson cell disruptor) in the presence of 5% trichloroacetic acid and ^{32}P labelled phospholipids were isolated by two-dimensional thin layer chromatography on silica gel 60 (Merk) using solvent systems described by Abdel-Latif *et al.*¹⁸. ^{32}P -phosphatidate content was calculated from the specific activity of ^{32}P in the incubation medium. To correct for differences in cell density between experiments the increase in ^{32}P -phosphatidate produced by carbachol or KCl was expressed as a per cent change relative to ^{32}P -phosphatidate in unstimulated cells. Values for ^{32}P -phosphatidate of unstimulated cells ranged between 0.27 and 2.3 pg atoms per aliquot cell suspension. Note that carbachol increased ^{32}P -phosphatidate accumulation nearly threefold within 10 s (mean and standard error calculated from four experiments), a time where contraction is not yet maximal. Exposure of cells to KCl, which also causes contraction with a time course similar to that produced by carbachol, failed to increase ^{32}P -phosphatidate accumulation. Change in ^{32}P -phosphatidate: ○, carbachol; ●, KCl; × contraction of carbachol.

increased Ca^{2+} levels produced by cholinergic stimulation. If this is so, then phosphatidate added exogenously should produce an increase in intracellular Ca^{2+} levels, hence contraction. Previous reports have shown that phosphatidate can elicit contractions in intact smooth muscle strips⁸, but such studies cannot rule out an indirect action (such as release of endogenous neurotransmitters). In isolated smooth muscle cells, phosphatidate at concentrations as low as 10^{-8} M produced contractions within 10 s. These contractions were equal in magnitude to those produced by carbamylcholine (Fig. 2).

As phosphatidate lies at an important branch point in glycerolipid metabolism, the ^{32}P labelling of phosphatidyl inositol noted by many investigators in other systems¹ may also arise as a consequence of increased ^{32}P -phosphatidate accumulation. The finding that carbamylcholine stimulates ^{32}P incorporation into phosphatidyl inositol in isolated smooth muscle cells subsequent to accumulation of ^{32}P -phosphatidate supports this contention⁴. It has previously been suggested that an increase in phosphatidyl inositol breakdown leading to an opening of 'Ca²⁺ gates' is the mechanism by which Ca^{2+} influx is accelerated by several

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agents^{1,9,10}. Although the present studies provide no information concerning the events leading to increased phosphatidate accumulation in response to carbamylcholine, it is likely that an accelerated hydrolysis of phospholipids, possibly including phosphatidyl inositol¹¹, is involved. However, the present studies define the time during which these events occur.

The way in which phosphatidate accumulation may initiate an increase in Ca^{2+} influx in the contractile response of smooth muscle to cholinergic agents can be inferred from other studies. As phosphatidate is an ionophore in artificial systems¹² it is reasonable to postulate a similar role in the cell membrane. The finding that phosphatidate monolayers are collapsed in the presence of Ca^{2+} , in effect removing the Ca^{2+} -phosphatidate complex from the interface, suggests the possibility that Ca^{2+} traverses the membrane complexed with phosphatidate, perhaps in the form of inverted micelles¹³. Furthermore, the accumulation of phosphatidate could increase membrane Ca^{2+} permeability without necessarily altering Na^+ or K^+ permeability since the affinity of phosphatidate for Ca^{2+} in aqueous media is 10^5 greater than that for monovalent cations¹⁴. Finally, if phosphatidate acts physiologically as an ionophore, then, to provide specificity in the control of membrane permeability, it would be expected to be ordered and segregated in regions of the membrane distinct from the bulk of zwitterionic phospholipids forming the bilayer. Numerous reports support the concept that phosphatidate forms electronegative domains, particles, or inverted micelles in membranes, especially in the presence of Ca^{2+} and basic proteins¹⁵⁻¹⁷. Thus, the association between changes in Ca^{2+} influx and changes in phosphatidate metabolism, noted in smooth muscle and many other tissues in

response to cholinergic and many other stimuli may be explained primarily by the generation of phosphatidate domains, functioning as Ca^{2+} 'channels'.

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Is phosphatidic acid a calcium ionophore under neurohumoral control?

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There has recently been renewed interest in a previously observed phenomenon¹ whereby hormones and neurotransmitters alter the rate of incorporation of ^{32}P -phosphate into phospholipids, specifically, phosphatidylinositol (PI) and phosphatidic acid (PA)². This has arisen from a theory formulated by Michell and coworkers which postulates that this 'phospholipid effect' is in some way intimately involved in the mechanism by which certain neurotransmitters and hormones activate membrane Ca gates²⁻⁵. However, although Michell's observations are consistent with a phospholipid involvement probably somewhere between receptor occupation and Ca gating, they do not suggest what the specific role of the phospholipids in the Ca gating mechanism might be. Recently, Salmon and Honeyman suggested⁶ that the critical event may be the net formation of PA following the net breakdown of PI which occurs in pancreas⁷, platelets⁸ and smooth muscle^{6,9}. PA has been shown to behave as a Ca ionophore in a Pressman chamber¹⁰, and thus it is possible that an increase in PA concentration in cellular membranes might increase the Ca permeability of those membranes (see also ref. 5). In the rat parotid, receptors associated with Ca gating (muscarinic, α -adrenergic and substance P) show a phospholipid effect whereas β -adrenergic receptors (which act on adenylate cyclase) do not^{11,12}. The phospholipid effect is Ca independent, and is not produced by the divalent cationophore A23187 (refs 11, 12). Also, an easily quantifiable response of this tissue (K efflux measured as release of ^{86}Rb) is absolutely dependent on the concentration of external Ca (ref. 13) and, by inference, on the magnitude of Ca influx¹⁴. We report here evidence suggesting that PA, which is formed during the reaction sequence of the phospholipid effect, may directly mediate the inward movement of Ca that results from activation of surface membrane receptors.

Levels of PA were measured in dispersed parotid cells¹⁵ with two-dimensional TLC¹⁶. Unstimulated cells were found to contain 1.24 ± 0.15 nmol per mg protein PA ($n = 6$). This was significantly ($P < 0.005$) increased to 2.83 ± 0.37 nmol per mg

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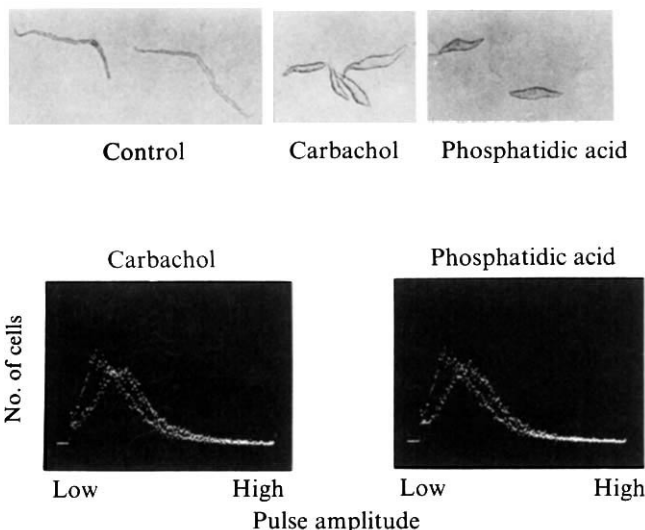


Fig. 2 Effects of exogenous phosphatidate on contractile state of isolated smooth muscle cells. Aliquots of single cell suspensions were exposed to 10^{-4} M carbamylcholine (carbachol) or 10^{-6} M phosphatidic acid (Sigma, grade I) for 15 s before fixation with acrolein⁷. Photomicrographs were taken at $\times 360$. The lower panel shows the pulse amplitude histograms obtained from 10^4 cells when aliquots of these same cell suspensions were analysed with a Coulter counter according to the method of Singer and Fay⁵. Note that both carbachol and phosphatidate produced contraction of the isolated cells such that cells length decreased by 60–70%. Phosphatidate-induced contraction was also noted when contractile state was assessed by Coulter counter analysis. Note that the pulse amplitude histogram generated by cells exposed to carbachol is shifted towards larger amplitude pulses when compared to the histogram generated by unstimulated cells. This increase in the number of large amplitude pulses is indicative of contraction of the isolated cells. A similar shift in the pulse amplitude histogram is noted for cells exposed to phosphatidate. In this experiment both carbachol and phosphatidate produced a 20% increase in the number of large amplitude pulses. In other experiments exposure of cells to phosphatidate at concentrations as low as 10^{-8} M or for as little as 5 s produced contractions of isolated smooth muscle cells.

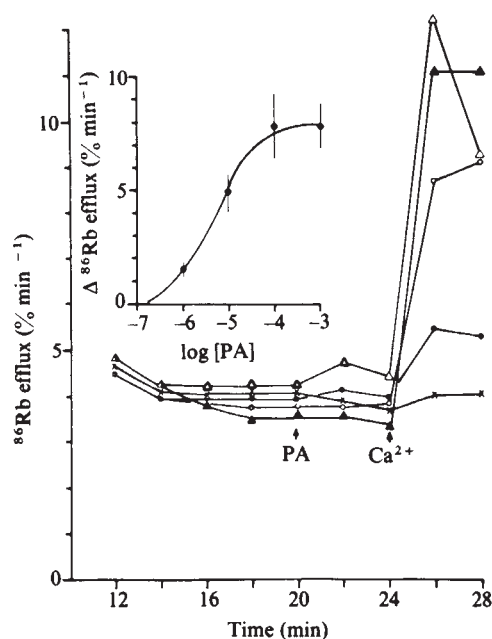


Fig. 1 Activity of phosphatidic acid (PA) in stimulating Ca-dependent ^{86}Rb release from rat parotid gland slices. The protocol of loading tissues with ^{86}Rb has been described elsewhere^{13,18}. The Ringer medium also contained 10^{-5} M atropine and 10^{-4} M phentolamine to prevent effects of endogenous neurotransmitter release on ^{86}Rb efflux. The Mg^{2+} concentration of this medium, and that for the experiments shown in Fig. 3, was 1.2 mM. This amount of Mg, about one-tenth of the K_a for Mg^{2+} , is not sufficient to interfere appreciably with Ca transport, but is necessary in the biological experiments to prevent membrane 'leakiness' that can occur on omission of extracellular Ca (ref. 22). Initially, the Ringer contained no added Ca and 10^{-4} M EGTA. At $t = 20$ min, phosphatidic acid was added to the media in the concentrations indicated, and at $t = 24$ min, CaCl_2 was added to a final concentration of 10 mM. Concentrations of PA (final) were: $\times$, none (control); $\bullet$, 10^{-6} M; $\circ$, 10^{-5} M; $\blacktriangle$, 10^{-4} M; $\triangle$, 10^{-3} M. The PA used was chromatographically pure ($\geq 99\%$), synthetic material (dipalmitoyl L- α -phosphatidate Na, Sigma P-4013). The values are means of three experiments each. The standard error averaged less than 10% of the means. Inset, basal ^{86}Rb efflux was subtracted and net increases in ^{86}Rb efflux at each concentration were calculated. Means of three experiments ± 1 s.e.m.

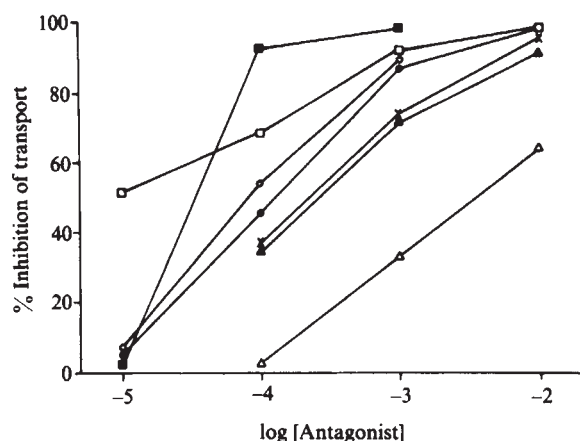


Fig. 2 Inhibition of PA-mediated ^{45}Ca transport into CHCl_3 by various agents. Equilibrium partitioning of ^{45}Ca (10^{-5} M) from 1.0 ml of Ringer medium (without Mg^{2+}) containing 10^{-4} M PA into 5.0 ml CHCl_3 was determined in the presence and absence of several agents at the concentrations indicated. The data are expressed as % inhibition; that is, 100% means no radioactivity in the CHCl_3 . The agents used were: $\square$, La^{3+} ; $\blacksquare$, Tm^{3+} ; $\circ$, neomycin; $\bullet$, Co^{2+} ; $\blacktriangle$, Ni^{2+} ; $\times$, Ca^{2+} ; $\triangle$, Mg^{2+} . The data summarise three separate experiments, each assayed in triplicate. The standard errors averaged 5% of the means.

protein PA ($n = 8$) on 30 min incubation with the muscarinic agonist, methacholine (10^{-4} M). Continued muscarinic receptor activation for this period of time produces a sustained activation of surface membrane Ca gates¹⁷.

Figure 1 shows the Ca-dependent stimulation of ^{86}Rb release from parotid gland slices by various concentrations of PA. Phosphatidylinositol and phosphatidylserine (another acidic phospholipid) had no effect at 10^{-4} M concentrations. Because the PA did not stimulate ^{86}Rb release until Ca was present, nonspecific effects on membrane permeability or an ability to transport ^{86}Rb directly can probably be ruled out. Also, as can be seen in Fig. 1, PA did not cause a transient response before the addition of Ca, as is typical for agents acting on endogenous receptors¹⁸. We conclude, therefore, that the action of exogenous PA is probably not mediated through endogenous receptors.

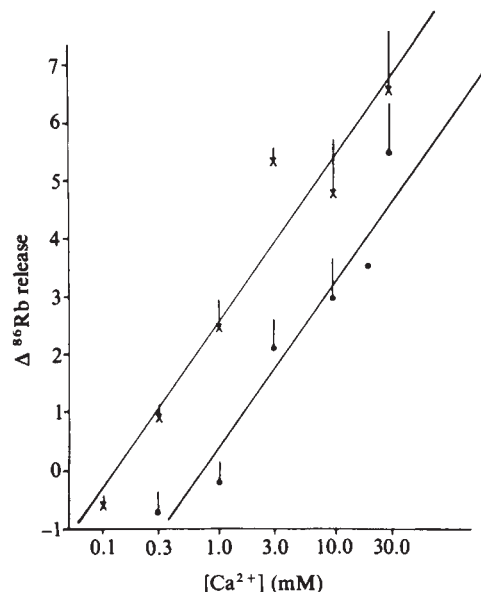


Fig. 3 Concentration-effect relationship for Ca-dependent ^{86}Rb release. The data were obtained from experiments similar in design to those shown in Fig. 1, except that the receptor-controlled Ca gating mechanism was activated with the muscarinic agonist, carbachol. To assure that Ca gating was rate limiting, a submaximal concentration of carbachol (10^{-6} M) was used¹⁸. Basal rates of ^{86}Rb release (before Ca was added) were subtracted. The linear portions of the curves were fitted by least-squares analysis, and the log-displacement of the curves at $y = 3\% \text{ min}^{-1}$ was used to calculate K_a values. $\times$, Control ($n = 4$); $\bullet$, plus 2 mM Co^{2+} ($n = 3$). The dispersions represent s.e.m.s. Other agents were tested with similar procedures and three or four replications each.

The assay of ^{45}Ca partitioning into CHCl_3 due to PA was carried out as follows: 1.0 ml of Ringer medium (with no Mg) containing 10^{-5} M ^{45}Ca and 10^{-4} M PA was shaken with 5.0 ml of CHCl_3 . After sufficient time for the system to come to equilibrium, the radioactivity in the organic phase was determined. In the absence of PA, virtually no radioactivity was detectable in the organic phase. With PA present, about 5% of the radioactivity originally added was extracted by the CHCl_3 . This effect was comparable to that seen with 20 μM A23187. Phosphatidylinositol and phosphatidylserine did not augment ^{45}Ca partitioning into CHCl_3 . Tyson *et al.*¹⁰ surveyed several phospholipids and found that only phosphatidic acid and cardiolipin produced this effect. The abilities of various agents to inhibit this process were determined by repeating the extraction in the presence of various concentrations of the appropriate reagents. The results are shown in Fig. 2. The relative potencies of the agents tested were in the order: $\text{La}^{3+} > \text{Tm}^{3+} > \text{neomycin} > \text{Co}^{2+} > \text{Ni}^{2+} \approx \text{Ca}^{2+} \gg \text{Mg}^{2+}$. Although the actual mechanism involved in the PA-induced partitioning of ^{45}Ca into

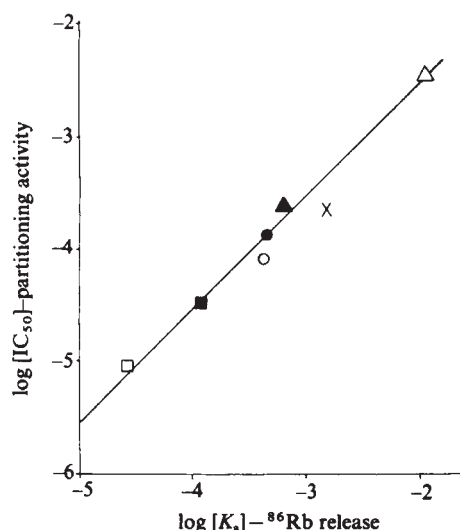


Fig. 4 Relationship between inhibition of ^{45}Ca -partitioning activity of PA and inhibition of receptor-activated Ca-dependent ^{86}Rb release by various agents. The ordinate values are concentrations of agent necessary to inhibit ^{45}Ca transport by PA into CHCl_3 by 50% as determined graphically from the data in Fig. 2. The abscissae are apparent dissociation constants (K_a) for antagonism of Ca gating in parotid slices determined as illustrated for Fig. 3. The agents were: $\square$, La^{3+} ; $\blacksquare$, Tm^{3+} ; $\circ$, neomycin; $\bullet$, Co^{2+} ; $\blacktriangle$, Ni^{2+} ; $\triangle$, Mg^{2+} ; $\times$, Ca^{2+} (K as agonist estimated). The line was fitted by least-squares analysis. Ca was omitted, however, because the K value, as an agonist, is somewhat uncertain. The slope (using logarithms) was 0.998 and the correlation coefficient was 0.993.

CHCl_3 is not known, it is reasonable to conclude that the potencies of these inhibitory cations in preventing this process reflects their ability to compete with ^{45}Ca for binding to the negatively charged head group of the PA. In addition, the Ca antagonist D-600, as well as procaine and tetracaine, were tested (not shown) and found to be somewhat less potent than Mg^{2+} .

The same agents were tested for their ability to inhibit endogenous, receptor-controlled Ca gating. The protocol here was to determine the Ca concentration-effect relationship for stimulating ^{86}Rb release (with carbachol as agonist) in the presence and absence of a fixed concentration of antagonist, and then to calculate antagonist dissociation constants from the shift in the Ca concentration-effect curve¹⁹. An example is shown in Fig. 3. The potencies for inhibition of Ca-mediated ^{86}Rb release (in the case of Ca, its apparent K value as agonist) ranked as $\text{La}^{3+} > \text{Tm}^{3+} > \text{neomycin} > \text{Co}^{2+} > \text{Ni}^{2+} > \text{Ca}^{2+} \gg \text{Mg}^{2+}$. Procaine, tetracaine and D-600 had no antagonistic activity below 10^{-3} M, and at higher concentrations produce inhibition by other mechanisms^{20,21}.

The correlation between inhibition of partitioning activity of PA and apparent affinity for the Ca influx mechanism (as assayed by ^{86}Rb release) is illustrated in Fig. 4. Taken with the observations that receptor activation increases the PA content of the parotid, and that exogenous PA can stimulate a Ca-dependent response in the parotid, these data suggest that PA may function in the physiological mechanism for Ca gating. This does not imply that PA must act in as simple a manner as for the ionophores in general; auxiliary structures and functional groups may also be involved. In red cells, for example, increased levels of PA do not apparently lead to augmentation of Ca influx⁵. As the red cell does not seem to have any Ca-gating mechanism under receptor control, this may indicate that other structures necessary for this phenomenon are also absent. However, in the parotid gland and other tissues, receptor mechanisms are present which regulate Ca gating as well as the membrane concentration of PA. On the basis of the data presented here, we suggest that the critical binding site for Ca

gating in these tissues could well be receptor-regulated phosphatidic acid.

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Motoneurone counts in *Xenopus* frogs reared with one bilaterally-innervated hindlimb

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The death of large numbers of young neurones is a striking feature in the developing nervous system. Many authors have postulated that the neurones are produced in excess of the numbers that their target tissues can support, forcing them to compete for survival¹⁻³. Observations on developing limb motoneurones (ventral horn cells) in both frogs and chicks seem to support this hypothesis. Normally, one-half to three-quarters of ventral horn cells die as limb movements begin^{4,5}, but some of these (up to 25% in chicks, 7% in frogs) can be rescued by the provision of supernumerary limb buds before motor axon outgrowth begins⁶⁻¹⁰. Conversely, amputation of the limb bud causes an increased death rate approaching 100% (refs 4, 11). The crucial test of the hypothesis depends on its prediction that the limb should limit the number of surviving motoneurones, and here I have attempted to make the test. For this, both sides of the spinal cord were forced to project to a single hind limb bud well before the onset of ventral horn cell death. It was found that the combined total of surviving motoneurones (right plus left sides) projecting to the single limb exceeded the number that projects to one limb in normal animals by up to ~100%. It is concluded that the limb does not limit the number of survivors, and therefore the hypothesis is refuted, at least for ventral horn cells. It now seems more likely that motoneurone numbers are determined either by the need to eliminate ventral horn cells not connected with appropriate limb regions, or by factors operating within the central nervous system.

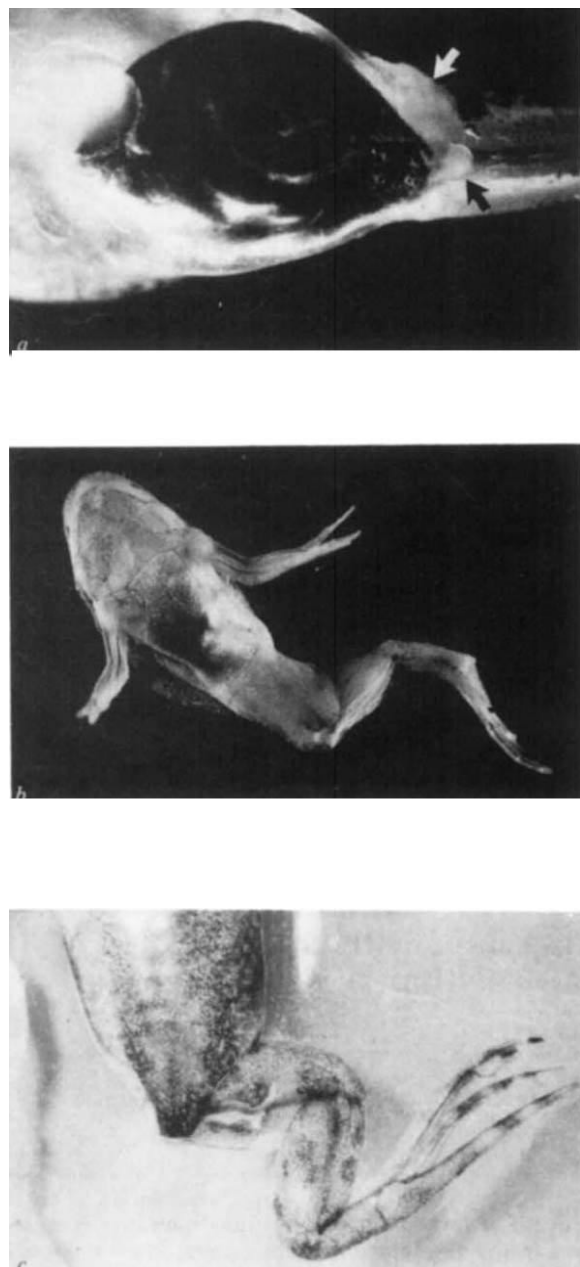


Fig. 1 *a*, Stage 49 tadpole (ventral view) after operation to move one limb bud (black arrow) into the midline. The other limb bud was first removed together with a small margin of surrounding epithelium. An incision was made through the tail fin caudal and dorsal to the anus and extended into the trunk along the dorsal surface of the peritoneal cavity, stopping just short of the renal duct. The flap of tail fin containing the anus (white arrow) was pulled across and fixed against the body wall with a fibrin clot to pull the remaining limb bud into the midline against the retroperitoneal wound. The flap returned to its normal position after 1 or 2 days, leaving the limb bud adhering to the new position. $\times 11$. *b*, Juvenile frog (M146) with a midline limb (ventral view). Photograph taken after fixation. This was the only experimental animal to have an abnormal limb, with two missing toes and a slightly reduced range of movement at the hip. $\times 2$. *c*, Juvenile frog (M213) without limb bud displacement (dorsal view). After amputating the other limb bud, a needle was used to tear the retroperitoneal region separating the two limb buds. Among the natural barriers to axon growth in this region are some large blood vessels which caused heavy bleeding. However, blood flow through nearby structures including the remaining limb seemed unaffected. $\times 7$. All operations were carried out in a solution containing 1 g sea salt, 4 g NaCl and 9 g l^{-1} D-glucose plus 1:5,000 MS222. Tadpoles recovered in a solution containing 5 g l^{-1} NaCl.

Operations were performed on 10–15-day-old *Xenopus laevis* tadpoles at stages 49 and 50 (ref. 12), when motor axon invasion of the limb buds is about to begin¹³. (Degeneration of ventral horn cells begins about a week later at stage 53, lasts 3–5 weeks until stage 60 and causes the loss of about 75% of the original population.) After removing one limb bud, the other was either left *in situ* or displaced into the midline, dorsal to the anus (Fig. 1*a*). Midline barriers which normally confine the spinal nerves to their own sides were disrupted with a needle. Only eight tadpoles survived the operations but they matured without delay. Limb buds regenerated in three tadpoles and were removed as they appeared.

In all except one frog with minor abnormalities (M146, Fig. 1*b*), the single hind limbs were normal in every respect including muscle bulk and amount and character of visually observable movements. None of the frogs had any trace of a limb, pelvic bones or muscles on the amputated side. To prove that in the metamorphosed frogs the limb was innervated by both sides of the cord, motoneurons projecting to the limb were labelled by retrograde axonal transport of horseradish peroxidase (HRP) either injected into the gastrocnemius muscle or applied to the proximal stump of the sciatic nerve after transection in the thigh¹⁴ (Fig. 2*a*). Further evidence of bilateral innervation was obtained at dissection 2 days later (7–14 days after completion of metamorphosis) by observing the spinal nerves from both sides entering the limb (Fig. 2*b*). The dorsal root ganglia were not preserved for cell counts but they seemed normal in size at dissection.

The numbers of motoneurons ipsilateral to the remaining limb were normal in all animals (Table 1). Contralateral motoneurone numbers varied from 11 to 94% of normal. Summing the two sides gives the total number of motoneurons projecting to the single limbs. In the cases with good bilateral innervation (over one-third contributed by the contralateral side) the totals ranged from 2,325 to 3,189, far exceeding the number of motoneurons projecting to one limb in control animals (range 927–1,704, Table 1). This result shows that the

Table 1

	Age at maturity (weeks)	Cell counts		Total	Left/right (%)
		Right (ipsi-lateral)	Left (contra-lateral)		
Experimental					
M188*	9	1,449	1,371	2,820	94
M146*†	10	1,668	1,521	3,189	91
M213†	7	1,353	1,155	2,508	85
M69	11	1,287	1,038	2,325	81
M208	7	1,617	1,119	2,736	69
M209	7	1,599	843	2,442	53
M68	11	1,140	321	1,461	28
M187*†	8	1,773	189	1,862	11
Controls					
Unoperated					
M160	8	1,458	1,497	2,955	103
M162	8	1,452	1,437	2,889	99
M163	8	1,632	1,572	3,204	96
Sham operated					
M167	8	1,440	1,404	2,844	98
M168	8	1,542	1,614	3,156	105
M169	8	1,587	1,521	3,108	96
Other operated					
M65	10	1,005	—	—	—
M66	10	927	—	—	—
M195	7	1,677	—	—	—
M198	7	1,704	—	—	—

Frogs M65–69, M146 and M160–213 were reared from three different batches of tadpoles of different parentage. Unoperated controls were raised in the same tank as M187 and M188. Sham-operated controls had midline barriers disrupted but no limb bud amputation. In two of these cases a few motoneurons were found to project contralaterally (2 and 16 contralateral HRP-labelled cells; one gastrocnemius injected). 'Other operated' controls had partial deletions of one limb bud at stage 50, but no disruption of midline barriers. Counts are from the unoperated sides.

* Limb bud displaced into the midline.

† Regenerating limb bud removed. All regenerates were less than 0.3 mm long on removal.

limb does not limit the number of motoneurons projecting to it at projection densities found in normal animals. It also seems unlikely that there is a limit at the higher densities achieved in this experiment. This is shown by the absence of any correlation between the proportion of the total (ipsilateral plus contralateral) motoneurone projection contributed by the contralateral side, and the ipsilateral motoneurone counts ($r = -0.073$).

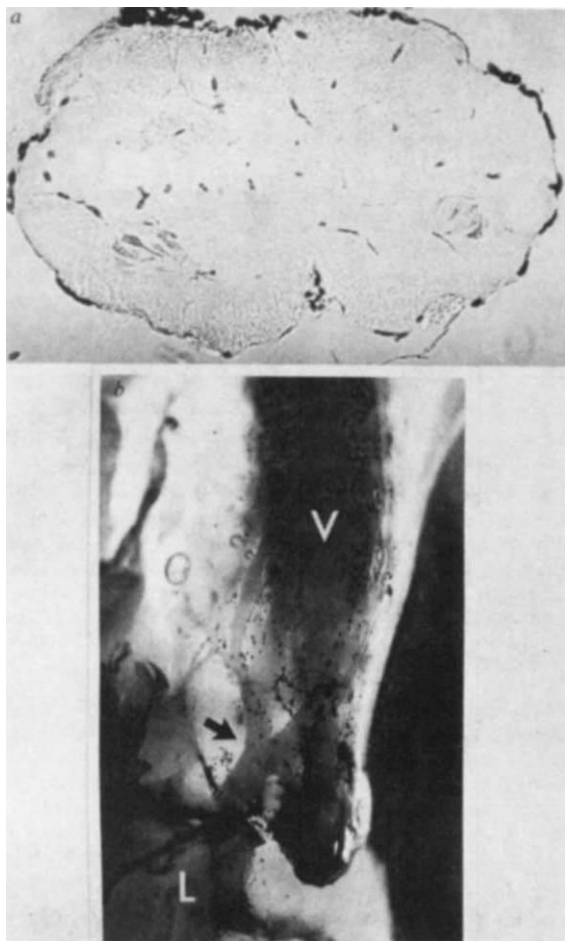


Fig. 2 Evidence for bilateral innervation. *a*, Transverse section of frog (M69) spinal cord showing HRP labelled motoneurons in both ventral horns. Bilateral labelling was also found in all other experimental animals except M146, which was not given HRP. In the case illustrated here, 30% HRP (Sigma VI) was applied to the transected sciatic nerve in the thigh. As in normal animals¹⁴, this method leaves about half the motoneurons unlabelled. Presumably, they project to the limb by other nerve trunks, or leave the sciatic nerve proximal to the transection. $\times 70$. *b*, Spinal nerves from both sides of the vertebral column (V) of M188 join to form one nerve trunk (arrow) before entering the base of the limb (L). $\times 14$. Spinal cords were dissected out and prepared for HRP histochemistry and cell counting by fixation in cold phosphate-buffered (pH 7.0) 2% glutaraldehyde for 4 h followed by overnight washing in running water. Cords were then immersed in 0.08% buffered diaminobenzidine tetrahydrochloride (Sigma) to which H_2O_2 had been added, after 1 h, to a concentration of 0.01%. Incubation at room temperature continued for 45 min before standard paraffin embedding for serial sections. Sections were mounted unstained in DPX and viewed by phase contrast microscopy. Counts were made immediately after mounting when cellular details, especially the nucleoli, are clearest. Motoneurons were identified using the criteria of Prestige⁴, which are based on position, alignment and size. Motoneurons with nucleoli were counted in every third section and no corrections were made for nucleolar splitting which probably occurs rarely⁴. Medial motor column neurones were not counted as they do not supply the limb. HRP labelling was assessed 2–3 weeks later after partial drying of the DPX to maximise visibility of the label.

Any competition should have shown as a negative correlation with ipsilateral counts falling as the contralateral projection increased. Conversely, the fact that ipsilateral counts were not increased above normal when the contralateral projection was small shows that the operation itself had not created a 'super-limb' with an increased motoneurone-supporting capacity.

Although it has been shown that the limb does not limit the number of survivors, a form of peripheral competition may still be postulated in which the survival rate is decided, not by the amount of space or trophic factors in the limb, but by a variable quality intrinsic to the ventral horn cells themselves. For example, using the analogy of territorial aggression, 'strong' ventral horn cells may acquire all the synaptic sites or trophic factors at the expense of the 'weak' cells. This idea was tested recently in *Xenopus* by allowing some ventral horn cells which normally die to have sole occupation of a part of the limb. Nevertheless, their deaths were not prevented and the part of the limb was left uninnervated¹⁵, proof that at least some cell deaths are not due to peripheral competition. This evidence and the present results militate strongly against any form of peripheral competition determining motoneurone numbers.

Although the limb may not directly control numbers it remains indisputable in both *Xenopus*⁴ and chick⁵ that ventral horn cells must make contact with it to survive. Indeed, concurrent experiments in *Xenopus* (unpublished) have shown that the contact must be made with regions of the limb for which the motoneurons have been specified. This may provide one explanation for the present result. Specification may be so precise that only the minority of ventral horn cells succeed in finding their region, but all those that do, survive.

Control of motoneurone numbers may also be exercised outside the limb. An intriguing possibility is that the ventral horn cells may yet be competing (for example, for afferent connections, blood supply) but in the spinal cord, not in the limb. Some authors have also conjectured that not all ventral horn cells are presumptive adult motoneurons capable of long-term survival. Apart from some work showing that all ventral horn cells have axons at least as far as the ventral root¹⁶, this question has not been studied in *Xenopus*. However, extensive investigations in the chick embryo have not so far shown any differences between ventral horn cells destined to live or die¹⁷.

We are left with the problem of how supernumerary limbs prevent some ventral horn cells from dying^{6–10}. The usual interpretation is that the increased limb field allows more competing cells to survive^{2,7}. With peripheral competition now in doubt, this interpretation is less attractive. Others should instead be considered more seriously, including effects of the supernumerary limbs on ventral horn cell specification¹⁸, or on the central control of their survival. Tests of these possibilities should be feasible.

Other interesting questions arising from this study concern the electrophysiology of the motoneurons projecting to the single limb, how they distribute their innervation to the muscle fibres and the implications for theories of axonal guidance. In the three frogs given HRP into the gastrocnemius, normal projections were found from both ventral horns. Further evidence of normal somatotopy would argue that at least in *Xenopus*, axons are actively guided into the limb and not just channelled fortuitously by mechanical or geographical factors as recent work in the chick embryo seems to suggest^{19,20}. The single limb preparation should be an excellent model for studying various aspects of developing limb innervation.

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Opiate receptors influence vasopressin release from nerve terminals in rat neurohypophysis

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A previous report described the existence of substantial amounts of enkephalin immunoreactivity and the occurrence of nerve terminals containing an enkephalin-like material in the pars nervosa of rat pituitary¹. It was suggested that an enkephalin innervation of the pars nervosa originating from the magnocellular hypothalamic nuclei might regulate the secretion of neurohypophyseal hormones. The results of the present studies support this hypothesis, as we find that a stable enkephalin analogue (D-Ala²,D-Leu⁵-enkephalin) inhibits the calcium-dependent release of vasopressin evoked by electrical stimulation of the rat pituitary stalk *in vitro*. A similar inhibition of the stimulus-evoked vasopressin release is caused by morphine and β -endorphin, and the inhibitory effects of the enkephalin analogue can be reversed by naloxone. These findings suggest the possible existence of inhibitory opiate receptors on the terminals of vasopressin fibres in the pars nervosa.

The release of vasopressin from isolated pars nervosa/pars intermedia tissue fragments was studied using a method similar to those previously described^{2,3}. The pars nervosa with attached pars intermedia tissue was dissected from adult male Sprague-Dawley rats (Charles River; 150–200 g) and incubated in 0.5 ml Krebs–bicarbonate medium at 37 °C continuously gassed with 95% oxygen:5% carbon dioxide. The pituitary stalk was drawn into the tip of a silver wire suction electrode to allow electrical stimulation and during periods of stimulation the gland was raised to the surface of the fluid. The medium was changed at

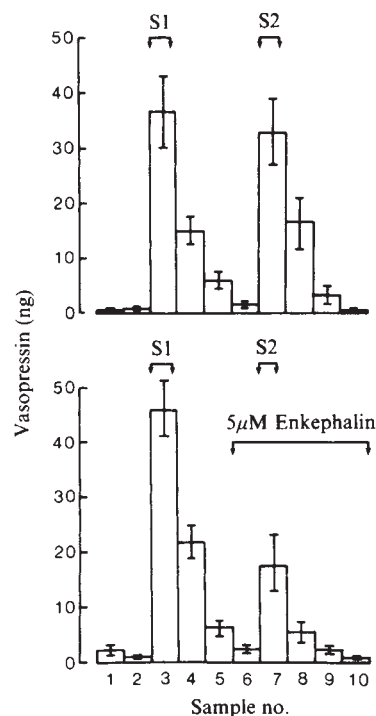


Fig. 1 Release of immunoreactive vasopressin from rat pituitary *in vitro* and inhibition by a stable enkephalin analogue. Rat pituitary pars nervosa/pars intermedia fragments were attached by the pituitary stalk to suction electrodes and incubated in Krebs–bicarbonate medium for 60 min before starting to collect sequential 15-min samples. During the experiment each gland was subjected to two periods of electrical stimulation (S1 and S2) through the pituitary stalk (30 V, 0.2 ms duration, 30 Hz in 10-s trains with 10-s intervals between trains for a total of 10 min). In 14 control experiments (a) the release of the vasopressin evoked during S2 was similar to that observed during S1. Addition of a stable enkephalin analogue (D-Ala²,D-Leu⁵-enkephalin = 5 μ M ENKEPHALIN) 15 min before S2 (b, eight experiments). Vasopressin was measured by radioimmunoassay and results are expressed as ng Arg⁸-vasopressin equivalents; each value is the mean $\pm$ s.e.m. for the number of experiments indicated.

15-min intervals and vasopressin was measured by a radioimmunoassay procedure, using ¹²⁵I-vasopressin (NEN), a rabbit antibody to vasopressin (Calbiochem-Behring) and synthetic Arg-vasopressin as standard. The immunoassay had a sensitivity limit of 0.2–0.4 pg per sample and there was no detectable cross-reactivity with oxytocin in amounts up to 200 ng. None of the test substances used had any effects on the vasopressin radioimmunoassay. Incubation samples were neutralised (10 μ l 0.2 M HCl) and stored frozen; they were diluted at least 10-fold with assay buffer before assay. As the spontaneous release of vasopressin was initially high, but subsequently declined to low levels, the first period of electrical stimulation was carried out after a preincubation period of 60 min. Phasic rather than continuous stimulation was chosen as this more closely mimics the natural firing pattern of the vasopressin neurones, and leads to an enhanced release of vasopressin *in vitro*⁴. In these conditions the resting release of vasopressin in 14 control experiments was 0.6 ± 0.13 ng Arg⁸-vasopressin equivalent in 15 min, corresponding to a spontaneous release rate of approximately 0.004% of total tissue content per min (tissue content at the end of experiment = $1,072.5 \pm 137.0$ ng vasopressin per gland, mean $\pm$ s.e.m., $n = 14$). Phasic electrical stimulation of the pituitary stalk (S1) led to an increase of more than 60-fold in vasopressin release, and a total of 51.2 ± 8.94 ng vasopressin (mean $\pm$ s.e.m., $n = 14$) was recovered in the stimulation sample and in the sample collected in the 15-min period after stimulation (Fig. 1). A second period of electrical stimulation (S2) 45 min after the first elicited almost the same release of vasopressin (49.1 ± 10.59 ng) as the first. In subsequent experiments,

Table 1 Calcium dependence and tetrodotoxin sensitivity of vasopressin release

	<i>n</i>	Arg ⁸ -vasopressin (pg equivalents per min)	
		Resting release	Stimulation-evoked release
Control	18	93 ± 24.7	$1,703 \pm 268.7$
Ca ²⁺ -free	4	140 ± 46.7	60 ± 7.7
Tetrodotoxin 1 μ g ml ⁻¹	4	127 ± 13.3	83 ± 18.0

Rat pituitary pars nervosa fragments were incubated for 45 min before collecting 15-min fractions to assay resting release of vasopressin and release evoked by electrical stimulation of the pituitary stalk (evoked release = average vasopressin release during 15 min stimulation sample +15 min post-stimulation period, see Fig. 1). Mean values $\pm$ s.e.m. for number of experiments (n) indicated.

therefore, test agents were introduced 15 min before the second period of stimulation, and results were expressed as the ratio of vasopressin released during the two stimulation periods (S2/S1).

Control experiments confirmed²⁻⁴ that the stimulation-evoked release of vasopressin was completely abolished by the addition of tetrodotoxin ($1 \mu\text{g ml}^{-1}$) or by removal of calcium ions from the incubation medium (Table 1). Addition of the stable enkephalin analogue D-Ala²,D-Leu⁵-enkephalin (DADLE)⁵ ($5 \mu\text{M}$) before the second stimulation period led to more than a 70% inhibition of the stimulus-evoked release of vasopressin (Fig. 1, Table 2), without any significant change in the spontaneous efflux of vasopressin. Similar inhibitory effects on the stimulation-evoked release of vasopressin were seen after addition of morphine ($10 \mu\text{M}$) or β -endorphin ($2 \mu\text{M}$). The inhibitory effect of DADLE was completely reversed by simultaneous addition of the opiate antagonist drug naloxone ($1 \mu\text{M}$) (Table 2). Naloxone alone, however, had no significant effect on the spontaneous or evoked release of vasopressin; γ -aminobutyric acid (GABA) ($50 \mu\text{M}$) and somatostatin ($1 \mu\text{g ml}^{-1}$) were also without effect (Table 2).

The results show that activation of opiate receptors in the rat neurohypophysis can inhibit the stimulus-evoked release of vasopressin from neurosecretory nerve terminals. These findings suggest that opiate receptors may exist on the terminals of vasopressin neurones in the pars nervosa, which possesses a high density of opiate binding sites⁶. The effects of opiates are selective, as two other biologically active compounds that occur in and can be released from the pars nervosa, GABA⁷ and somatostatin⁸, had no effects on vasopressin release. The present findings are at first sight difficult to reconcile with several previous reports which suggest that the administration of opiates or endorphins *in vivo* leads to an enhanced release of vasopressin from the pituitary⁹⁻¹². However, this general conclusion has been questioned¹³, and recent studies have reported that systemically or centrally administered opiates and endorphins can lead to a reduction in vasopressin immunoreactivity in rat plasma^{14,15}. In addition to the inhibitory effects on vasopressin release from neurohypophysis reported here, opiates may act centrally to inhibit the firing of vasopressin neurones (ref. 16 and G. Clarke and P. Wood, personal communication). Furthermore, the present results are complementary to those of Clarke *et al.*¹⁷, who recently reported that intraventricularly administered morphine led to a suppression of oxytocin release from rat pituitary, and suggested the existence of opiate receptors on oxytocin-containing nerve terminals in the neurohypophysis. Our findings confirm a previous report that β -endorphin failed to affect the spontaneous release of vasopressin from rat pars nervosa *in vitro*¹², although these authors did not examine the

effects of endorphin on the stimulus-evoked release of vasopressin. The concept that opiate receptors may exert an inhibitory influence on the release of a biologically active substance from nerve terminals agrees with other studies which have shown that opiate receptors in other parts of the nervous system control the release of acetylcholine¹⁸, noradrenaline¹⁸ and substance P¹⁹. The possible physiological function of such a control mechanism in the neurohypophysis remains to be defined.

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Multiple opiate receptor sites on primary afferent fibres

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In addition to their actions at supraspinal sites, opiates can act directly at the spinal cord level to produce analgesia¹⁻³. Opiate receptors and enkephalins are found in abundance in the dorsal horn of the spinal cord, in the region of termination of small-diameter primary afferents⁴⁻⁶. Furthermore, there is evidence that exogenously administered opiates or endogenous enkephalins act both on postsynaptic receptors in dorsal horn and presynaptically to block transmitter release from nociceptive primary afferent terminals⁷⁻¹⁰. The possibility of these two distinct sites of opiate action in dorsal horn is of particular interest in view of recent evidence for the existence of multiple opiate receptors¹¹⁻¹³. On the basis of differences in the rank orders of potency of opiate alkaloids and opioid peptides observed in different tissues, Lord *et al.*¹⁴ have proposed two categories of opiate receptor, μ and δ , and these can be differentiated in binding studies, provided that selective radioligands are used in the appropriate experimental conditions¹⁵⁻¹⁸. Here we have used the selective radioligands ³H-morphine (μ sites) and ³H-D-Ala²,D-Leu⁵-enkephalin (δ sites) to examine directly in rat the distribution and binding characteristics of opiate receptors on dorsal root and in various regions of the adjacent spinal cord. Primary afferent tissue (dorsal root) and dorsal horn were found to contain μ and δ opiate binding sites with a relatively high proportion of μ sites. Partial destruction of small-diameter primary afferents after cutting the sciatic nerve led to a significant reduction in both μ and δ binding sites on dorsal roots, suggesting that both types of opiate receptors may exist on small-diameter primary afferents.

Table 2 Effect of opiates and other substances on stimulation-evoked release of vasopressin from rat pars nervosa

	n	S2/S1 (mean $\pm$ s.e.m.)
Control	14	0.99 $\pm$ 0.12
D-Ala ² ,D-Leu ⁵ -enkephalin ($5 \mu\text{M}$)	8	0.28 $\pm$ 0.08*
Morphine sulphate ($10 \mu\text{M}$)	5	0.28 $\pm$ 0.10*
β -Endorphin ($2 \mu\text{M}$)	3	0.44 $\pm$ 0.14*
D-Ala ² ,D-Leu ⁵ -enkephalin ($5 \mu\text{M}$) + naloxone ($1 \mu\text{M}$)	7	1.06 $\pm$ 0.26
Naloxone ($1 \mu\text{M}$)	5	0.97 $\pm$ 0.24
Somatostatin ($1 \mu\text{g ml}^{-1}$)	3	0.98 $\pm$ 0.18
GABA ($50 \mu\text{M}$)	3	1.14 $\pm$ 0.29

After a control period of electrical stimulation (S1), test substances were added to the incubation medium 15 min before a second period of electrical stimulation (S2). Results are expressed as the ratio S2/S1, representing the total amounts of vasopressin released (during the stimulation period and in post-stimulation sample) by the two stimulation periods. Values are means $\pm$ s.e.m. for the number of experiments indicated (n).

* $P < 0.01$ compared with control.

Using a modification of the approach described by Chang and Cuatrecasas¹⁸, commercially available high-specific activity tritium-labelled morphine and D-Ala²,D-Leu⁵-enkephalin at low concentrations were chosen as selective radioligands for μ and δ opiate binding sites, respectively^{17,18}. As described by Lord *et al.*¹⁴, μ receptors are characterised by their high affinity for morphine and related alkaloids and for the antagonist drug naloxone, whereas δ receptors preferentially recognise enkephalins and are relatively resistant to naloxone. Preliminary experiments were carried out using rat corpus striatum, a region of the central nervous (CNS) system known to contain a high density of both μ and δ binding sites¹⁷. There was a considerable amount of specific binding (defined by the use of 1 μ M levorphanol) of both radioligands in membrane preparations from striatum; at low concentrations of each ligand (≤ 1 nM) specific binding accounted for 80–90% of total binding. Saturation analyses over the concentration range of 0.1–15.0 nM for ³H-D-Ala²,D-Leu⁵-enkephalin indicated the presence of a single binding component on Scatchard plots, with an apparent K_d of 2.4 nM (Table 1). Similar analyses for ³H-morphine suggest the existence of both low- and high-affinity binding components, but over a restricted range of low concentrations of ligand (0.2–1.5 nM) a single high-affinity component predominated, with an apparent K_d of 1.2 nM. The potency of various opiates, opiate antagonists and opioid peptides in competing for ³H-morphine or ³H-D-Ala²,D-Leu⁵-enkephalin binding was determined, using the approach described by Terenius and Wahlström¹⁶ in which a low concentration of radioligand (0.5 nM) was combined with the same concentration of the second unlabelled ligand to

Table 1 Properties of ³H-morphine and ³H-D-Ala²,D-Leu⁵-enkephalin binding in rat striatum and spinal cord

	Striatum		Spinal cord	
	³ H-morphine	³ H-D-Ala ² , D-Leu ⁵ - enkephalin	³ H-morphine	³ H-D-Ala ² , D-Leu ⁵ - enkephalin
Apparent K_d (nM $\pm$ SEM)	1.2 $\pm$ 0.08	2.4 $\pm$ 0.18	1.0 $\pm$ 0.28	3.9 $\pm$ 0.64
	IC ₅₀ values (nM)			
D-Ala ² ,D-Leu ⁵ - enkephalin	15.0	3.8	16.0	7.6
Met ⁵ -enkephalin	4.0	2.6	—	—
Leu ⁵ -enkephalin	5.2	1.0	—	—
Levorphanol	1.0	11.0	0.9	2.1
Morphine	2.5	240.0	3.0	68.0
Naloxone	1.8	34.0	3.5	14.0
Diprenorphine	0.4	0.16	—	—
Ethylketocyclazocine	3.4	7.4	—	—

Corpus striatum or whole spinal cord were dissected from adult male Sprague-Dawley rats and homogenised in 50 vol ice-cold 50 mM Tris-HCl buffer, pH 7.4 (Polytron, setting 7, 15 s). The homogenate was centrifuged (4°C, 50,000g, 15 min) and resuspended in 50 vol Tris buffer (Polytron, setting 7, 15 s). After incubation for 45 min at 37°C, to digest endogenous enkephalins, membrane fragments were collected by centrifugation and suspended in ice-cold Tris buffer at a final concentration (in terms of original tissue wet weight) of 10 mg ml⁻¹ (striatum) or 20 mg ml⁻¹ (spinal cord). Samples (1 ml) of the membrane suspension were incubated with ³H-morphine ([1(n)-³H]morphine; specific activity 27 Ci mmol⁻¹; Radiochemical Centre) or ³H-D-Ala²,D-Leu⁵-enkephalin (D-Ala²,D-Leu⁵-[tyrosyl-3,5-³H]enkephalin; specific activity 48 Ci mmol⁻¹; Radiochemical Centre) for 20 min at 30°C. The labelled membranes were collected by rapid filtration on Whatman GF/B glass fibre filter disks, and washed with 2 $\times$ 5 ml of Tris buffer. After soaking the filters for at least 2 h in 4 ml ethoxyethanol, radioactivity was measured by liquid scintillation counting after addition of 10 ml scintillation fluid. Nonspecific binding was defined by parallel incubations in the presence of 1 μ M levorphanol. Incubations and non-specific blanks were always carried out in triplicate. The protein content of individual membrane preparations was determined by the method of Lowry *et al.* To avoid losses of ³H-enkephalin due to adsorption to glassware and pipettes the stock solution of the labelled peptide was diluted in plastic tubes with Tris buffer containing bovine serum albumin (Sigma; crystalline grade) 0.1 mg ml⁻¹ in preparing a stock solution; this was further diluted 1:500 (v/v) in the final incubations. IC₅₀ values were determined at 0.5 nM radioligand concentration as illustrated in Fig. 1. K_d values were determined by Scatchard analysis of specific binding measured in triplicate at 4–6 ligand concentrations in the concentration range 0.1–15.0 nM for ³H-D-Ala²,D-Leu⁵-enkephalin, and 0.2–1.5 nM for ³H-morphine.

Table 2 Distribution of opiate binding sites in rat spinal cord, striatum and spinal roots

	Specific binding (fmol per mg protein)		
	³ H-morphine	³ H-D-Ala ² , D-Leu ⁵ - enkephalin	Binding ratio
Dorsal root	8.9 $\pm$ 0.45(7)	2.7 $\pm$ 0.22(6)	3.2 $\pm$ 0.22(6)
Dorsal horn	8.1 $\pm$ 0.66(4)	4.9 $\pm$ 0.79(4)	1.7 $\pm$ 0.28(4)
Ventral horn	3.6 $\pm$ 0.56(4)	3.6 $\pm$ 0.20(4)	1.0 $\pm$ 0.11(4)
Ventral root	2.2 $\pm$ 0.31(3)	<1.0(3)	—
Sciatic nerve	<1.0(5)	<1.0(5)	—
Corpus striatum	24.0 $\pm$ 2.54(7)	44.2 $\pm$ 2.69(7)	0.5 $\pm$ 0.04

Spinal tissues were removed following laminectomy in stunned decapitated rats. Roots were dissected from L4–L6 ganglia to cord entry, sciatic nerve from notch to knee. Each cord half was divided at the level of the canal into dorsal and ventral parts. Each measurement included pooled tissue from 6 to 10 adult male rats (250–250 mg tissue). All measurements were made at 0.5 nM concentrations of radioligand. Specific binding, defined using 1 μ M levorphanol, was 40–60% of total for ³H-morphine and 20–45% for ³H-D-Ala²,D-Leu⁵-enkephalin. In some cases the specific binding of radioligands was less than 1 fmol per mg protein and was considered to be below the limit for accurate measurement (<20% specific binding). Using the Student's *t*-test, the amount of ³H-morphine binding in ventral horn differed significantly from that in dorsal horn ($P < 0.005$) and dorsal root ($P < 0.001$). For the binding ratios, dorsal horn differed significantly from dorsal root ($P < 0.01$) and ventral horn ($P < 0.01$). Other comparisons did not show significant differences. Statistical comparison with ventral root and sciatic nerve were not made. Values are means $\pm$ s.e.m. for the number of experiments indicated in brackets. The binding ratio represents binding of each radioligand at a single concentration (0.5 nM) and gives a relative indication of the proportions of μ and δ binding sites.

ensure that drug potencies were compared in conditions of similar basal receptor occupancy.

The results obtained with the various drugs tested (Fig. 1, Table 1) were similar to those reported by Chang and Cuatrecasas¹⁸, who used ³H-dihydromorphine or ³H-naloxone and ¹²⁵I-D-Ala²,D-Leu⁵-enkephalin. Provided the tritium-labelled compounds were used at concentrations below their K_d values (0.5 nM), they seemed to serve as selective ligands for μ and δ opiate receptors, respectively. Morphine itself was the best discriminating drug, as it was ~100 times more potent in competing for the binding of ³H-morphine (μ sites) than for ³H-D-Ala²,D-Leu⁵-enkephalin (δ sites, Fig. 1). Naloxone was ~20 times more potent on μ than on δ sites, and nonradioactive D-Ala²,D-Leu⁵-enkephalin, Met-enkephalin and Leu-enkephalin were more potent on δ than on μ sites. Because neither ³H-morphine nor ³H-D-Ala²,D-Leu⁵-enkephalin are completely selective ligands for μ and δ sites, saturation analysis cannot provide accurate values for the absolute numbers of μ and δ sites in particular regions of the CNS. However, the use of these radioligands at low concentrations provides a useful index of the ratio of μ to δ sites. The present results suggest a preponderance of enkephalin (δ) receptors over morphine (μ) receptors in corpus striatum.

By using homogenates of whole rat spinal cord, binding sites for both radioligands could be detected with K_d values and inhibitor sensitivities similar to those observed in striatum (Table 1). Although the density of binding sites in spinal cord was much lower than in striatum, there was significant binding of both the peptide (δ) and alkaloid (μ) agonists on pooled samples of lumbosacral dorsal roots and in dorsal and ventral horn. Binding sites for either ligand were barely detectable on ventral root or peripheral nerve (Table 2). Compared with striatum, where δ binding predominated (with a μ/δ ratio of about 0.5), there was relatively more μ binding in dorsal root and dorsal horn, with μ/δ binding ratios of 3.2 and 1.8, respectively (Table 2). To determine whether the ³H-enkephalin (δ) binding in dorsal roots represented a possible cross-reactivity of this ligand with μ receptors, displacement studies were carried out with nonradioactive morphine. Addition of 25 nM nonradioactive morphine caused an 85% inhibition of ³H-morphine binding in

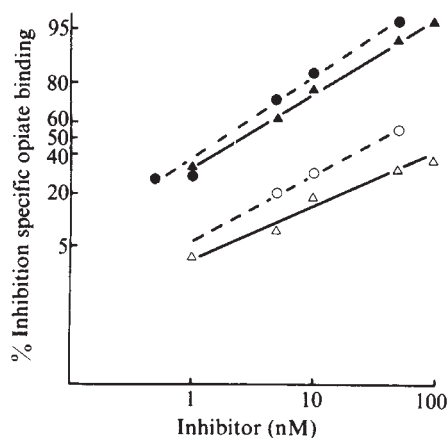


Fig. 1 Determination of IC_{50} values for morphine (triangles) and naloxone (circles) as inhibitors of specific binding of 3H -morphine (solid circles and triangles) and 3H -D-Ala,D-Leu-enkephalin (open circles and triangles), each at 0.5 nM in rat striatal membrane preparation. Each point represents the mean of triplicate determinations. All tubes for 3H -morphine binding measurements also contained 0.5 nM nonradioactive D-Ala,D-Leu-enkephalin, and tubes for 3H -enkephalin binding contained 0.5 nM nonradioactive morphine to ensure similar basal receptor occupancy¹⁶. Ordinate: per cent inhibition of specific 3H -labelled radioligand binding (defined by 1 μ M levorphanol) probit scale; abscissa: log concentration of nonradioactive morphine or naloxone.

dorsal root samples, but less than a 10% inhibition of 3H -D-Ala,D-Leu-enkephalin binding ($n = 2$). Because of the small amounts of dorsal root tissue obtainable, it was not possible to carry out detailed saturation analyses or complete drug displacement curves. However, the results clearly demonstrate that both types of opiate site are present on primary afferent fibres. Furthermore, the observation that binding ratios for the two types of agonist differ significantly on dorsal root and dorsal horn supports the idea that there are separate pre- and post-synaptic populations of opiate receptor, with a relatively greater proportion of δ -type receptors on postsynaptic elements in the dorsal horn.

To determine whether the opiate binding sites are present on small-diameter primary afferent fibres, further experiments were carried out after cutting the sciatic nerve. There is evidence that this procedure leads to a loss predominantly of small-diameter afferent fibres in the dorsal roots¹⁹⁻²¹. In three groups of 10 rats, the sciatic nerve was cut 1 month before dissection of the L5 and L6 dorsal roots, which were pooled to prepare membranes for ligand binding studies. The results showed a significant reduction in both μ and δ binding sites on the dorsal roots after sciatic nerve section (Table 3).

The present results provide the first direct evidence that primary afferents have significant numbers of opiate receptor sites on their central processes. This conclusion is consistent with previous findings of a depletion of opiate binding sites in dorsal horn following damage to primary afferents^{22,23}. Autoradiographic evidence has also shown that the central processes of vagus nerve afferents, which are predominantly unmyelinated, bear opiate receptors²⁴. Furthermore, administration of capsaicin to neonatal rats causes a relatively selective loss of small-diameter primary afferents²⁵ and this procedure leads to a

reduction of opiate receptors in dorsal horn^{26,27}. These findings are consistent with neurophysiological evidence that opiates can act directly on the terminals of small-diameter primary afferent fibres^{28,29} and exert a selective inhibitory effect on nociceptive input to dorsal horn neurones^{9,10}, adding further support to the hypothesis that opiates may act presynaptically to block pain transmission at the spinal cord level.

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Changes in composition and activity of microtubule-associated proteins during brain development

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The onset of neuronal differentiation is characterised by intensive neurite growth; because microtubule formation is strictly required during this process¹⁻³, *in vitro* assembly of the tubulin present in the rat brain has been studied at different stages of development⁴: the rate of assembly is very slow in the early stages and increases progressively with age from birth until adulthood. Other data⁴ also suggested that the limiting factor in the young brain is the amount or activity of one or several of the minor components which co-polymerise into microtubules with tubulin. We show here that both the composition and the activity of the microtubule-associated proteins change during the time course of rat brain development.

Rat brain supernatants were prepared at two stages of post-natal development, 3 days after birth (that is, at the beginning of the critical period of development in this species) and at day 35 (near adulthood). The tubulin present in both supernatants was allowed to assemble *in vitro* in the conditions described by

Table 3 Reduction of μ and δ binding in dorsal roots 1 month following sciatic cut

	Specific binding (fmol per mg protein)	
	3H -morphine (μ)	3H -D-Ala,D-Leu-enkephalin (δ)
Normal control (Table 2)	8.9 $\pm$ 0.45(7)	2.7 $\pm$ 0.22(6)
Sciatic cut	5.9 $\pm$ 1.18(3)	1.3 $\pm$ 0.05(3)
% Reduction	-34%	-51%

The sciatic nerve was cut 30 days before removal of the dorsal roots (L5, L6). The cut side significantly differed from control ($P < 0.05$ for μ , $P < 0.005$ for δ).

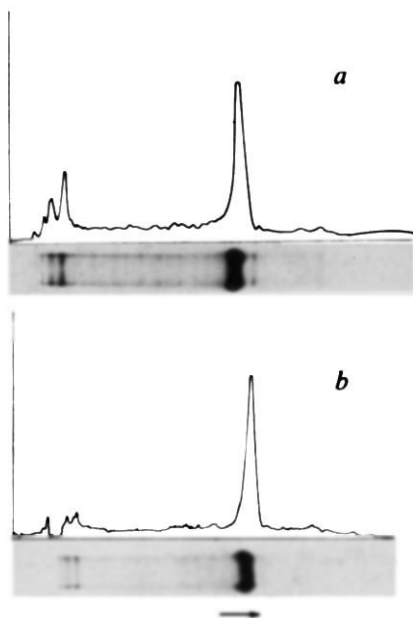


Fig. 1 Analysis by SDS gel electrophoresis of microtubule proteins prepared from adult (a) and 3-d-old (b) rat brain. Microtubules were obtained by the procedure described by Shelanski *et al.*⁵ slightly modified⁶. The microtubule pellets were depolymerised by cold in the presence of NaCl 0.75 M. Samples of these preparations were made up with 1% SDS and 1% dithiothreitol and then heated to 100 °C for 5 min. Aliquots of these samples containing 11 µg of microtubule proteins were electrophoresed according to the method of Weber and Osborn⁷ on a slab gel with a 4–15% gradient of polyacrylamide. The gels stained with Coomassie blue are represented for the two preparations of microtubule proteins.

Shelanski *et al.*⁵ slightly modified⁶. At the end of the incubation period the microtubules were pelleted by centrifugation, washed and analysed by polyacrylamide gel electrophoresis in the conditions described by Weber and Osborn⁷. Fig 1a and b, show that, in addition to tubulin, several minor components are present in both 'young' and 'adult' preparations. Among these associated proteins one group with high molecular weight (~300,000) contains two components similar to MAP₁ and MAP₂ (or HMW₁ and HMW₂) first described by Murphy and Borisy^{8,9}. The young preparation contains both MAP₁ and MAP₂; several analyses show that the percentage of these entities does not differ from that measured in adult microtubules (MAP₁ + MAP₂/tubulin = 16% in the young preparation and 17.5% in the adult one). Another group of MAPs, not well separated from tubulin and similar to the τ protein complex first isolated by Weingarten *et al.*¹⁰, is seen in the microtubules of both ages. The τ complex is thermostable¹⁰, and so, to study better this group of microtubule-associated proteins at both ages, purification was achieved as previously described¹¹ by thermic treatment of the *in vitro* assembled microtubules. Tubulin and MAP₁ were eliminated during this treatment and the other microtubule-associated proteins could be analysed at a much higher concentration by polyacrylamide gel electrophoresis. Figure 2a, which represents the analysis of the adult associated proteins, shows the presence of MAP₂ and the τ complex with its characteristic four closely spaced bands. With the young preparation MAP₂ is clearly present (Fig. 2b), but the pattern at the level of the τ complex is completely different: only two bands are present and none of them seem to be identical to any of the four bands present in the adult preparation. Identical results were found in four different experiments; moreover, after a second cycle of assembly the same protein pattern was found at the level of the τ complex, only two bands for the young preparation, with none of them being similar to any of the four closely spaced bands of the adult one. In addition, a control experiment was

carried out in the presence, throughout the procedure, of a protease inhibitor (phenylmethylsulphonyl fluoride) to rule out the possibility that the changes seen at the level of the τ complex are due to proteolytic breakdown of this or other (MAP₁, MAP₂)¹² fractions in the young preparation. Polyacrylamide gel electrophoresis revealed no marked differences in the protein patterns.

The activity of young and adult microtubule-associated proteins preparations in promoting tubulin assembly was also assayed. Pure adult tubulin was prepared by phosphocellulose chromatography^{11,13}; as previously shown, such tubulin does not assemble *in vitro* into microtubules even at high concentrations^{8,10,11,14,15}. A constant amount of pure tubulin was then incubated at 37 °C in the presence of increasing concentrations of both young and adult associated proteins obtained by thermic treatment; Fig. 3a shows the time course of assembly. Clearly, the activity of the young microtubule-associated proteins was much lower than that of the adult one. Fig. 3b represents the relationship between initial rates of assembly and the concentration of young or adult microtubule-associated

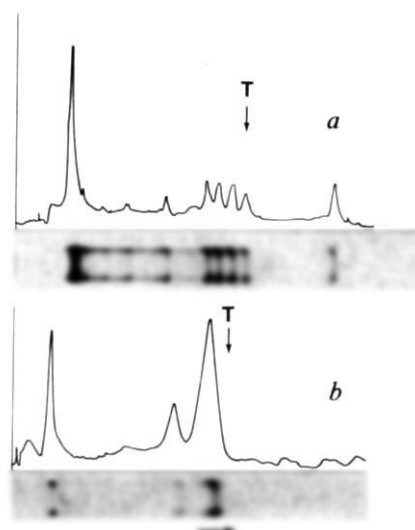


Fig. 2 Analysis by SDS gel electrophoresis of thermostable microtubule-associated proteins (or MAPs) prepared from adult (a) and 3-d-old (b) rat brain. Thermostable MAPs were prepared by a thermic denaturation procedure previously described¹². Samples of MAPs containing 7 µg for 3-d-old rat brain preparation and 11 µg for adult rat brain preparation were electrophoresed in the conditions described in Fig 1 legend. The gels stained with Coomassie blue are represented for the two kinds of MAP preparations. T, Position of tubulin.

protein preparations. It can be seen that (1) the rate of assembly increases linearly with both preparations and remains always much lower for the young one, and (2) the minimal concentration of microtubule-associated proteins at which assembly may be observed (critical concentration¹⁶) is higher with the young than the adult preparation. Similarly, we have previously shown that the critical concentration of tubulin is higher with young crude brain supernatants than with adult ones⁴.

Because young microtubule-associated proteins contain MAP₂, which is active¹⁷ in promoting *in vitro* microtubule assembly, but not the normal τ fraction distribution, one may speculate that the activity present at the early stage is due to MAP₂ only. Conversely, one or both of the two peaks migrating in the τ region might have some activity. Purification of the slow and fast components present in the young preparation is in progress so that their activity may be tested separately.

From the data reported here, a model may be proposed to explain the role of increasing neurotubule assembly during brain development. Large pools of tubulin would be synthesised before the building up of the neuronal network^{18,19}; some or all

of this tubulin would be used at earlier stages of development to allow the formation of the mitotic spindle and therefore proliferation of the undifferentiated nerve cells. At the beginning of the differentiation phase, specific proteins with polymerising activity would appear in increasing amounts, thereby allowing fast microtubule assembly and intensive neurite growth. Thus, the transition between cell division and cell differentiation might be controlled, at least in part, by an increase in the polymerising activity or by qualitative changes in the factors required for

either pathway or both. Although further work is needed to decide on the requirements for microtubule assembly before nerve cell differentiation, our data support the idea that the appearance of specific factors is responsible for the increase in the rates of microtubule assembly during the critical period of brain development—during (and for) intensive neurite growth.

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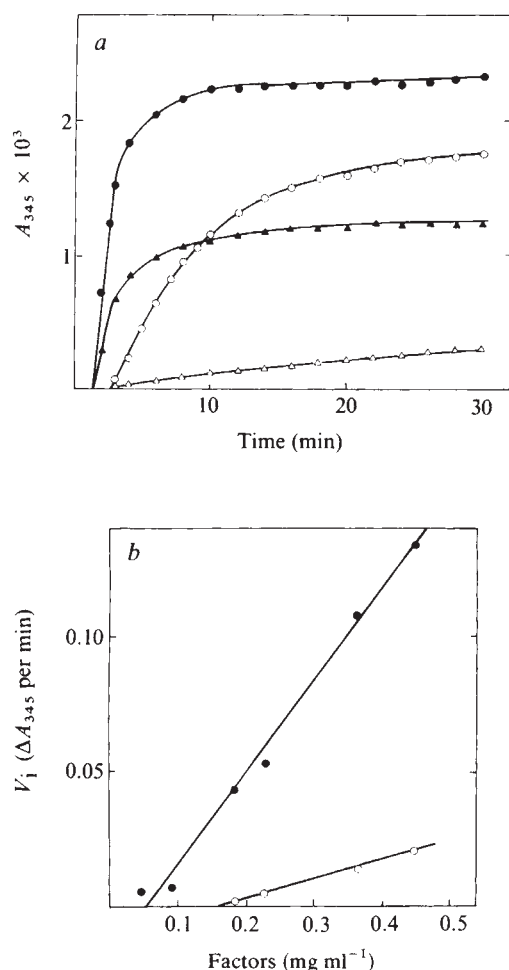


Fig. 3 Polymerisation-promoting activity of adult and 3-d-old rat brain thermostable microtubule-associated proteins on purified tubulin. Purified tubulin was obtained by a procedure previously described by Weingarten *et al.*¹⁰ but slightly modified: activated phosphocellulose was resuspended in the assembly buffer containing (in mM) morpholinoethane sulphonate 100, MgCl_2 0.5, EGTA 1, EDTA 0.1, GTP 1 and 2-mercaptoethanol 1, pH 6.4. Tubulin was eluted from the column with the same buffer. The purity of the tubulin was controlled by SDS gel electrophoresis. The polymerisation-promoting activity of the two kinds of MAPs on purified tubulin were followed by the turbidimetric method described by Gaskin *et al.*¹⁶. Turbidimetry measurements (345 nm) during tubulin polymerisation, at 37 °C, in the assembly buffer previously described, were carried out using a Carl Zeiss PM 6KS spectrophotometer with an automatic thermostated four-sample changer. *a*, Time course of tubulin polymerisation in the presence of the two preparations of thermostable microtubule-associated proteins. Pure tubulin (0.95 mg ml^{-1}) was incubated in the presence of two concentrations of thermostable MAPs: 'adult' MAPs ($\blacktriangle$, $181 \mu\text{g ml}^{-1}$; $\bullet$, $452 \mu\text{g ml}^{-1}$) and '3-d-old' MAPs ($\triangle$, $181 \mu\text{g ml}^{-1}$; $\circ$, $452 \mu\text{g ml}^{-1}$). *b*, Relationship between initial rates of tubulin polymerisation and thermostable MAPs concentration. Initial rates of tubulin polymerisation (ΔA_{345} per min) were measured with increasing concentrations of adult MAPs ($\bullet$) and 3-d-old MAPs ($\circ$) and a constant tubulin concentration (0.95 mg ml^{-1}).

In vivo release of acetylcholinesterase in cat substantia nigra and caudate nucleus

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Acetylcholinesterase (AChE), which is known to inactivate acetylcholine (ACh), is present in great abundance in the substantia nigra, although ACh levels and choline acetylase activity in this region are relatively low¹. Nigral dopaminergic cell bodies and their dendrites also contain AChE²⁻⁴. The functional significance of this enzyme in nigro-striatal dopaminergic neurones has been questioned^{1,4,5}. Earlier studies demonstrated an evoked release of AChE from unidentified central neurones into cerebrospinal fluid (CSF) in cats⁶, rabbits^{7,8} and dogs⁹. Later experiments have provided indirect evidence that the substantia nigra may contribute to a substantial amount of AChE detected: a unilateral nigral lesion in rabbits reduced AChE levels in the CSF, whereas electrical stimulation of the substantia nigra induced the opposite effect¹⁰. To investigate the possible release of AChE from dopaminergic dendrites and terminals we measured the *in vivo* release of this enzyme from the substantia nigrae and caudate nuclei of cats implanted with four push-pull cannulae and compared it with that of dopamine (DA). DA is released from dendrites in the substantia nigra^{11,12} as well as nerve terminals in the caudate nucleus. Spontaneous AChE release was observed in the substantia nigra and in the caudate nucleus. Moreover, the application of potassium (30 mM) in one substantia nigra increased the local release of AChE. This was accompanied by remote changes in the enzyme release from the other three structures which differed from that seen for DA. The different patterns of responses observed for AChE and DA suggest that AChE may also originate from other neurones in both the substantia nigra and the caudate nucleus.

Push-pull cannulae were acutely implanted in the two caudate nuclei and substantia nigrae of halothane-anaesthetised cats as described previously¹¹. The four structures were superfused with artificial CSF at a rate of 1.2 ml h⁻¹ and the activity of AChE^{13,14}, nonspecific cholinesterase^{13,14}, and occasionally lactate dehydrogenase¹⁵, were estimated in successive 10-min fractions. In a second series of experiments the release of ³H-DA continuously synthesised from L-[3, 5-³H]-tyrosine (50 Ci mmol⁻¹, 50 µCi ml⁻¹) was estimated in the four structures¹⁶. In all cases, the stereotaxic placement of the tip of each push-pull cannula was verified histologically.

A spontaneous release of AChE, which remained constant throughout the experiment, was observed both in the substantia nigrae and in the caudate nuclei. The amount of AChE released was similar in both structures (Fig. 1). Nonspecific cholinesterase was also observed in superfusates, the levels being slightly higher than those of AChE in the substantia nigrae and in the caudate nuclei (Fig. 2).

The addition of potassium chloride (30 mM) for 10 min to the artificial CSF in the left substantia nigra markedly increased the local release of AChE, the maximal change being seen during the treatment. Surprisingly, this effect was accompanied by changes in AChE release in the three other structures. Whereas AChE release was also increased in the contralateral caudate nucleus, it was decreased in the ipsilateral caudate nucleus and the contralateral substantia nigra (Fig. 1). These distal changes were more sustained than those observed in the left substantia

nigra. In contrast, the release of nonspecific cholinesterase was not affected in the left substantia nigra or the other three structures (Fig. 2). As cat plasma contains a substantial amount of nonspecific cholinesterase (269 ± 47 mU ml⁻¹, *n* = 5) as well as AChE (254 ± 44 mU ml⁻¹, *n* = 5), the unchanged levels of nonspecific cholinesterase in superfusates would exclude a plasma origin for the observed changes in the release of AChE. Furthermore, the local release of AChE induced by potassium cannot be attributed to gross cell damage as the activity of lactate dehydrogenase (a cytoplasmic enzyme) in the superfusates remained unchanged during potassium application (before, 1.5 ± 0.6 mU ml⁻¹; after, 1.5 ± 0.6 mU ml⁻¹; *n* = 4). Therefore, the release of AChE is a specific process.

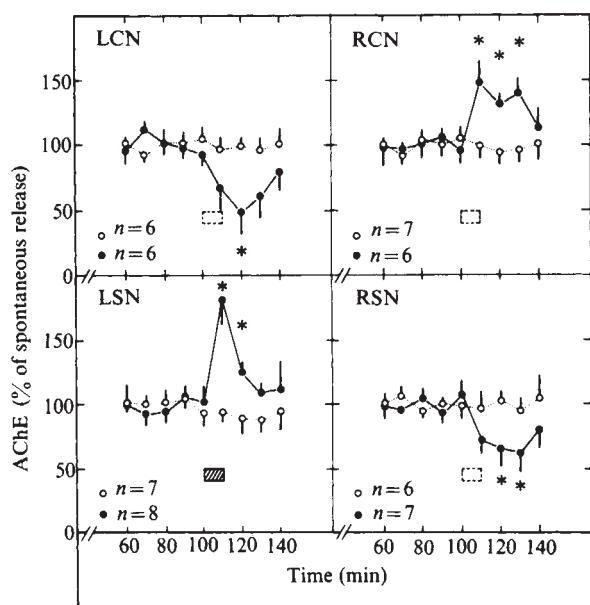


Fig. 1 Effects of the unilateral nigral application of potassium (30 mM) on the release of acetylcholinesterase (AChE) in both substantia nigrae and caudate nuclei. Four push-pull cannulae were simultaneously implanted in the left (LCN) and the right (RCN) caudate nuclei and in the left (LSN) and the right (RSN) substantia nigrae in anaesthetised cats. The four structures were superfused with an artificial CSF (1.2 ml h⁻¹) and AChE was estimated in 10-min successive superfusate fractions 60 min after the beginning of the superfusions. Potassium chloride (30 mM) was applied for 10 min in the LSN (hatched box with solid sides). The empty boxes with dotted sides represent the time during which potassium chloride (30 mM) was applied in the LSN. In each animal, and for each cannula, AChE in each successive fraction was expressed as a percentage of an average spontaneous release calculated from the five fractions collected (1.5 ± 0.4, 1.8 ± 0.5 and 1.7 ± 0.3, 1.6 ± 0.5 mU ml⁻¹ in the left and right substantia nigra and caudate nucleus respectively). Data are the mean ± s.e.m. of results obtained with treated animals (●). *P* < 0.05 when compared with corresponding control values obtained in untreated animals (○).

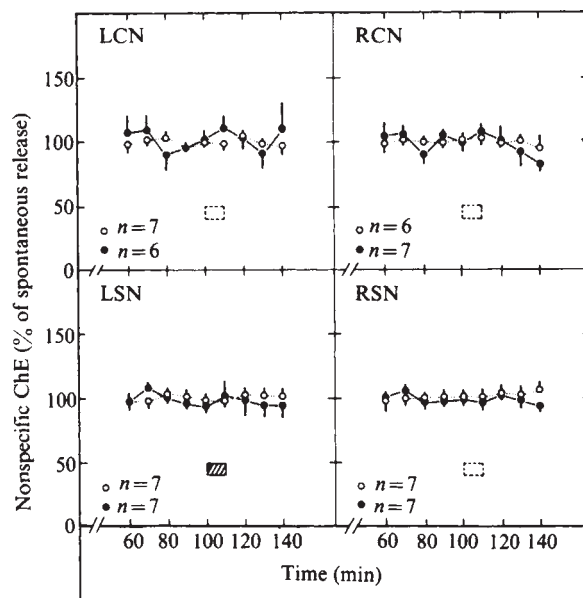


Fig. 2 Lack of effect of the unilateral nigral application of potassium (30 mM) on the release of nonspecific cholinesterase (ChE) in both substantia nigrae and caudate nuclei. During the experiments described in Fig. 1, we also measured nonspecific ChE in 10-min superfusate fractions and expressed the results as described in Fig. 1. The mean spontaneous release of nonspecific ChE in the left and right substantia nigra and in caudate nucleus were 2.3 ± 0.5, 2.7 ± 0.7 and 2.4 ± 0.5, 2.6 ± 0.6 mU ml⁻¹, respectively. Data are the mean ± s.e.m. of results obtained with control (○) and treated cats (●). Boxes as in Fig. 1.

Studies on peripheral nerves and innervated tissue have indicated that AChE can be released through a process dependent on nerve activity^{17,18}. The distal changes in AChE release observed in the present study suggest that this phenomenon also occurs in the central nervous system (CNS). There is, therefore, little doubt that the release of AChE in the substantia nigrae and caudate nuclei has a physiological significance.

If AChE originates from nigro-striatal dopaminergic neurones, the simplest hypothesis is that AChE should behave as does DA released from dendrites and nerve terminals in the substantia nigra and caudate nucleus respectively. We thus measured the release of newly synthesised ³H-DA in the four structures. Confirming previous results, the unilateral nigral application of potassium (30 mM) enhanced the release of ³H-DA in both caudate nuclei, the effect being slightly less pronounced in the contralateral structure¹⁹. Moreover, potassium increased the release of ³H-DA not only locally in the left substantia nigra but also, surprisingly, in the right substantia nigra. Therefore, the nigral application of potassium induced opposite effects on AChE and ³H-DA release in the ipsilateral

caudate nucleus and the contralateral substantia nigra. These findings do not exclude the possibility of a release of AChE from dopaminergic dendrites and nerve terminals. However, in this case, the messages reaching the ipsilateral caudate nucleus or the contralateral substantia nigra and responsible for the release of AChE and DA would have to be mediated by different pathways, or the two compounds would have to display a differential response to the same signal. Another hypothesis is that AChE originates additionally or exclusively from non-dopaminergic neurones.

Although histochemical and biochemical studies have clearly indicated that a high proportion of AChE is present in dopaminergic cell bodies and their dendrites, nigral AChE also has another location. As shown in the rat substantia nigra, 6-hydroxydopamine lesions, which reduced tyrosine hydroxylase activity by at least 90%, decreased that of AChE by merely 43% (ref. 4). Furthermore, nigral AChE was only reduced by 44% after a local lesion with kainic acid, a neurotoxin which completely destroyed the dopaminergic neurones and is also known to induce the degeneration of other nigral neurones²⁰. AChE not contained in dopaminergic neurones does not originate from striato-nigral fibres, as a kainic acid lesion in the striatum failed to affect nigral levels of AChE²¹. Therefore, afferent fibres from other regions may be involved. The situation is even more complex in the striatum, as in the rat, the dopaminergic terminals only contain about 12% of the entire striatal AChE⁴. Furthermore, kainic acid lesions in the striatum

showed that about 50% of the enzyme was distributed in intrinsic neurones²¹. Therefore, as initially suggested by Shute and Lewis²², various afferent nerve fibres could be rich in AChE. In fact, electrolytic lesions of the centro-median nucleus of the thalamus reduced AChE as well as choline acetyltransferase activities by 50% in the cat²³. Hence, AChE measured in superfusates of the substantiae nigrae and the caudate nuclei could originate from sites other than dopaminergic dendrites or terminals.

Previously, we demonstrated the existence of complex mechanisms involved in the reciprocal control of the activity of the two nigro-striatal dopaminergic pathways. Asymmetric changes in DA release were observed in both caudate nuclei after modifications of DA transmission in one substantia nigra²⁴, or after unilateral sensory stimulation²⁵. These were associated with opposite asymmetric changes in the dendritic release of DA in both substantiae nigrae (reminiscent of the changes in AChE release, Fig. 1). In contrast, symmetric responses in DA release were seen in the two caudate nuclei during modifications of GABAergic or glycinergic transmission in one substantia nigra¹⁹. The existence of two different polysynaptic pathways was postulated to account for the asymmetric and symmetric effects observed¹⁹. Surprisingly, in the same experimental condition, that is, the unilateral nigral application of potassium, two opposite patterns of response for AChE and DA release were seen in the four structures, one being asymmetric (AChE) and the other symmetric (DA). This indicates once more that various neuronal pathways are involved in the reciprocal regulation of the activity of neurones innervating the two substantiae nigrae and caudate nuclei.

Although the origin of AChE released from the substantia nigra and the caudate nucleus has still to be elucidated, these results demonstrate that AChE can be released in the CNS through processes involving nerve activity. It has been suggested that AChE could have a role other than that of inactivating ACh^{1,5}. Before this hypothesis can be verified, it must be established whether or not the distal changes in the release of AChE induced by the unilateral nigral application of potassium are associated with changes in cholinergic transmission. Finally, whatever the mechanisms are in the control of AChE release from both substantiae nigrae and caudate nuclei, they should be taken into account in our subsequent understanding of neuronal interactions within the basal ganglia.

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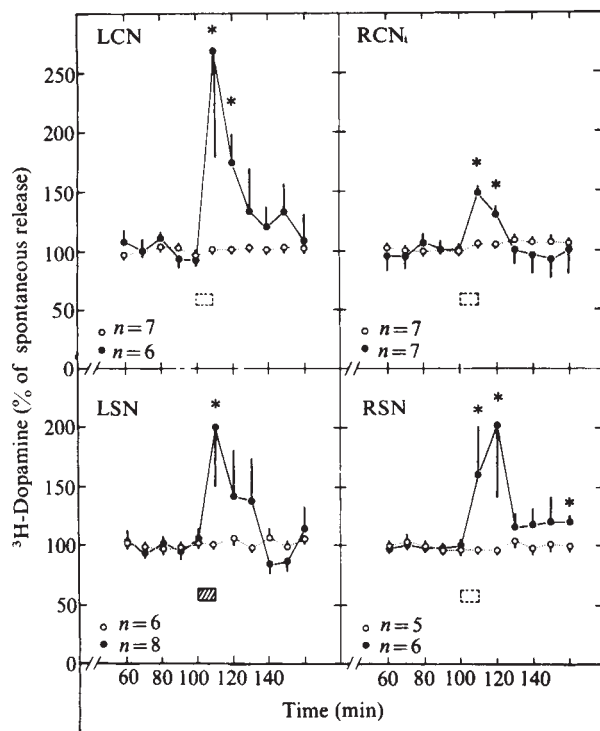


Fig. 3 Effect of the unilateral nigral application of potassium (30 mM) on the release of ³H-DA from the two substantiae nigrae and the two caudate nuclei. The experimental paradigm was essentially the same as described in Fig. 1, except that the artificial CSF delivered to the four push-pull cannulae contained L-[3,5-³H]tyrosine (50 Ci mmol⁻¹, 50 μ Ci ml⁻¹, 1.2 ml h⁻¹) and that ³H-DA was estimated in 10-min successive superfusate fractions. Results were expressed as described in Fig. 1. The mean spontaneous release of ³H-DA in the left and right substantiae nigrae and left and right caudate nuclei being 0.65 ± 0.07 , 0.57 ± 0.05 and 0.71 ± 0.08 , 0.82 ± 0.10 nCi per 10 min, respectively. Data are the mean $\pm$ s.e.m. of results obtained with treated animals (●), $P < 0.05$ when compared with corresponding control values (○).

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Glycocalyx is not required for slow inward calcium current in isolated rat heart myocytes

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The importance of the slow inward calcium current (I_{si}) in the excitation-contraction coupling process of cardiac muscle is well documented^{1,2}. The current can be attributed mainly to a calcium translocation from the extracellular space into the cell or a subsarcolemmal compartment of it^{3,4}. I_{si} has been suggested to have its source in and to be controlled by the surface coat of the sarcolemma (glycocalyx)⁵. The glycocalyx is destroyed in myocytes dissociated from adult heart tissue with solutions containing low calcium, collagenase and hyaluronidase^{6,7} (Fig. 1). By comparing the I_{si} data obtained in isolated myocytes with those reported for trabeculae or papillary muscles, we have now obtained evidence suggesting that the glycocalyx is not important in the genesis of I_{si} .

Whereas low calcium solutions are necessary for the dissociation of the cells, analysis of I_{si} requires superfusion with a Tyrode solution containing 2–4 mM calcium. The cells were isolated using the method described in ref. 8. Ventricles were minced into 3-mm coarse fragments and incubated in a solution composed of 130 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 11 mM glucose, buffered with 5 mM HEPES-NaOH to pH 7.4 and containing 1 mg ml⁻¹ collagenase (Sigma, Type C 2139) and 1 mg ml⁻¹ hyaluronidase. The cells were separated from the supernatant at 30-min intervals; the remaining tissue fragments were reincubated. The Tyrode solution contained 150 mM NaCl, 5.4 mM KCl, 3.6 mM CaCl₂, 1 mM MgCl₂ and 10 mM glucose, buffered with 5 mM HEPES-NaOH to pH 7.4. On return from low to physiological calcium concentrations rat heart muscle cells are susceptible to the 'calcium paradox' (ref. 9): the cells develop a strong contracture, become spherical and finally die. This problem was overcome by incubating the isolated cells in high K⁺, substrate-enriched solutions before stepwise elevation of calcium to a final concentration of 3.6 mM. Preincubation was at room temperature for 40 min in solution A (0.5 mM EGTA, 2 mM ATP, 2 mM succinate, 2 mM pyruvate, 2 mM β -hydroxybutyrate, 11 mM glucose, 20 mM taurine, 1.2 mM MgSO₄ and 100 mM K₂HPO₄, adjusted with KH₂PO₄ to pH 7.4) and for 20 min in solution B (1 mM CaCl₂ = 0.3 mM free Ca²⁺; 2 mM ATP, 2 mM succinate, 2 mM pyruvate, 1.2 mM MgSO₄, 100 mM KCl and 30 mM K₂HPO₄, adjusted with KH₂PO₄ to pH 7.4).

In about 50% of the rod-shaped cells we measured resting potentials between -75 and -85 mV, values similar to those reported previously^{10,11}. The cells did not beat spontaneously but they contracted in response to a stimulus. The action potentials (Fig. 2) resembled those of intact rat heart ventricular tissue. Voltage-clamp experiments were carried out with a single microelectrode technique (see Fig. 3a). The membrane current

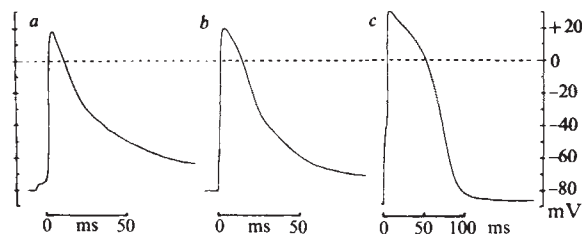


Fig. 2 Rat heart action potentials, intact tissue (a) compared with isolated myocytes (b, c). Isolated cells were viewed through an inverted microscope and impaled with 3 M KCl-filled microelectrodes. The configuration of the action potential differed appreciably: some cells displayed short-lasting action potentials (b) similar to those recorded from the intact trabecular (a), other cells displayed action potentials with a well pronounced plateau. Recordings from 20 cells revealed durations between 60 and 110 ms and amplitudes between 105 and 130 mV. All records were obtained in oxygenated Tyrode solution containing 3.6 mM CaCl₂. Temperature was 37 °C, stimulation frequency 1 Hz. Signals were digitised (10 bit per word) with a 2 K memory and stored on a digital tape recorder (ERDAC macrodyn).

evoked by a step depolarisation from -50 to 0 mV always started with a large initial outward surge and then declined within 10–12 ms to a minimum before reaching a steady outward level (Fig. 3b, see also Figs 4, 5). I_{si} was separated from the net current in the usual way^{3,4,12}. We subtracted the current waveform from the currents measured at the end of the clamp step and plotted this difference on a semi-log scale (Fig. 3d, ●). For clamp periods longer than 15 ms the plot shows a single exponential decay ($\tau_i = 10$ ms) which we associated with the inactivation of I_{si} . τ_i increased when the membrane was clamped to more positive potentials (Fig. 5c).

When inactivation of I_{si} was subtracted, the remaining currents changed during the first 15 ms of a clamp step with multiple time constants. An analysis of these currents (for time course of I_{si} activation) seemed impossible because of the limitations of the single electrode clamp technique (compare Fig. 3a).

An estimate of the voltage dependence of I_{si} amplitude was obtained by taking the difference in current between the quasi-steady state and the negative peak¹². The corresponding I - V curve (Fig. 4b, ●) reached a maximum at about 0 mV and crossed the V axis at a reversal potential of +50 mV ($+55 \pm 5$ mV; mean $\pm$ s.e. from 5 cells). The compound D600 (ref. 13) greatly reduced I_{si} at all potentials (Fig. 4b, ×).

The voltage dependence of the steady-state activation (d_{∞}) and inactivation (f_{∞}) variables was analysed with the classical^{3,4} conditioning step program (Fig. 5a, b). The activation curve d_{∞} started between -50 and -40 mV, increased e -fold per 22 mV (16 and 18 mV for two other cells) and reached saturation at 0 mV. Steady-state inactivation (f_{∞}) appeared between -40 and -30 mV, changed e -fold over 12 mV (11 and 12.5 mV for two other cells) and was complete around 0 mV. The curves intersect between -50 and 0 mV, suggesting a steady state I_{si} within this potential window.

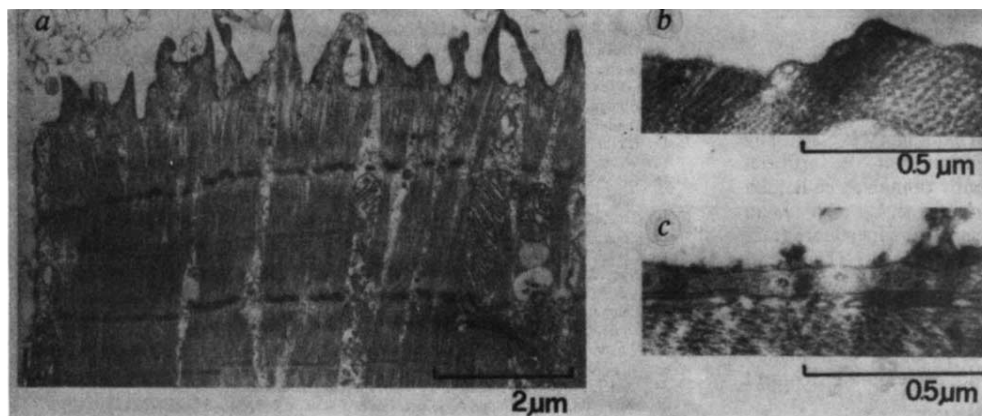
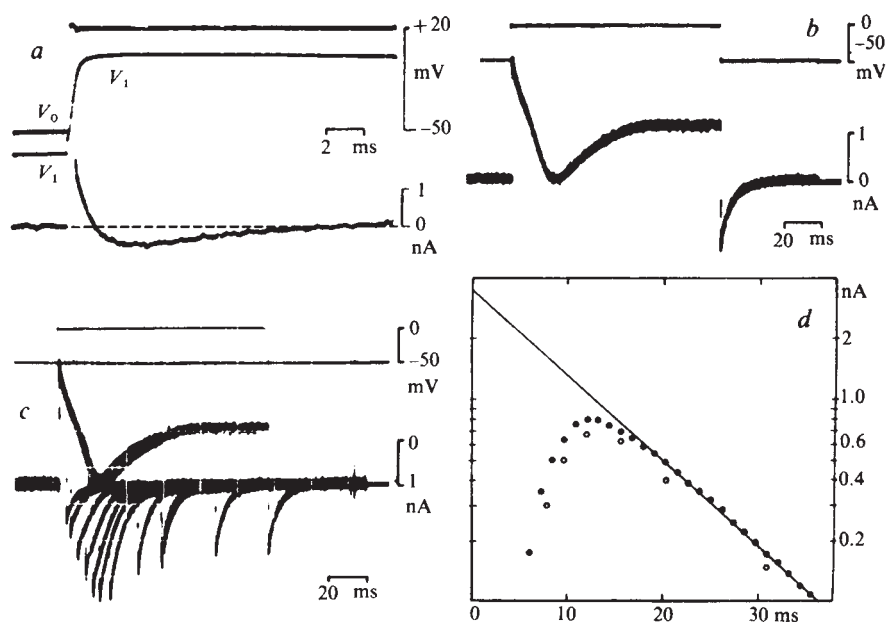


Fig. 1 Transmission electron micrographs of myocytes isolated from adult rat hearts and superfused with Tyrode solution. a Shows a longitudinal section of a myocyte separated at the intercalated disk. The normal arrangement of the myofilaments and the mitochondria are typical of unassociated heart muscle. The transverse section b shows a structural intact sarcolemma; no glycocalyx or other associated surface structures are present. Transverse section c was taken for comparison from non-dissociated rat heart tissue; here the sarcolemma is superimposed by a glycocalyx shown as a lanthanum-stained lamina about 70 nm thick. Specimens were handled by a convenient technique^{6,7}. For staining the surface coat¹⁷ (b, c) Tyrode solution containing 2 mM LaCl₃ superfused the preparations 30 min before fixation. In addition, four samples were stained with ruthenium red; again (as in b), no glycocalyx was present at the surface of the isolated myocytes (not illustrated).

Fig. 3 Voltage-clamp experiments carried out with a single intracellular microelectrode. An electronic current pump¹⁸ injected current and simultaneously measured the membrane potential, electronic compensation minimised the current-induced voltage drop at the electrode resistance which was reduced by bevelling to 2.5 M Ω . (It has been shown¹⁹ that currents 2 nA through bevelled microelectrodes produce no tip polarisation or rectification.) *a*, Evaluation of the method. A second (current-free) microelectrode was impaled 50 μ m from the site of current injection; it measured the potential V_1 . Whereas V_0 , the potential of the current-injecting electrode followed the command within 0.2 ms, V_1 shows a delay of about 2 ms. Afterwards, V_1 is stable and deviates from V_0 by -2.5 mV. *b*, Membrane currents in response to a depolarising clamp step. The holding potential of -50 mV inactivated the fast sodium current before the 120-ms step to 0 mV. The validity of this method is indicated by the finding that I_{si} remained constant when tetrodotoxin was added (5×10^{-5} g l $^{-1}$) or Na $^+$ was replaced by tetramethylammonium or Tris. *c*, After having depolarised the cell



for different periods of time, repolarisation back to -50 mV evoked an inward-directed tail current with an amplitude which reflects the conductance change at 0 mV. The envelope of the tails follows the same time course as the net current recorded at 0 mV (see Fig. 4a). *b* and *c* were photographed from an oscilloscope in storage mode. *d*, A semi-log plot of the difference between the current waveform and the current level at the end of the depolarising clamp step. The current record shown in *b* is analysed with the filled circles, the envelope of the tail currents (*c*) with the open circles.

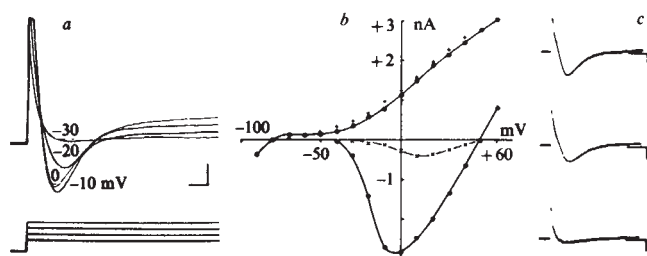


Fig. 4 (Above) Dependence of membrane currents on clamp potentials. The current tracings (*a*) were resolved into i_∞ (outward current measured at the end of the 200-ms pulse which is not shown) and I_{si} (peak negative current minus i_∞); values were plotted against the potential of the clamp step (circles in *b*). Exposure to 2 μ M D600 for 3 min ($\circ$, $\bullet$, before; +, $\times$, after application) strongly reduced I_{si} ($\times$) but not i_∞ (+). Currents evoked by a step to 0 mV before, 1 and 2 min after exposure to 2 μ M D600 are compared in *c*. Interference from the fast sodium current was minimised by imposing all voltage steps from a holding potential of -50 mV. Calibration marks in *a* and *c* indicate 10 ms and current from 0 to -1 nA.

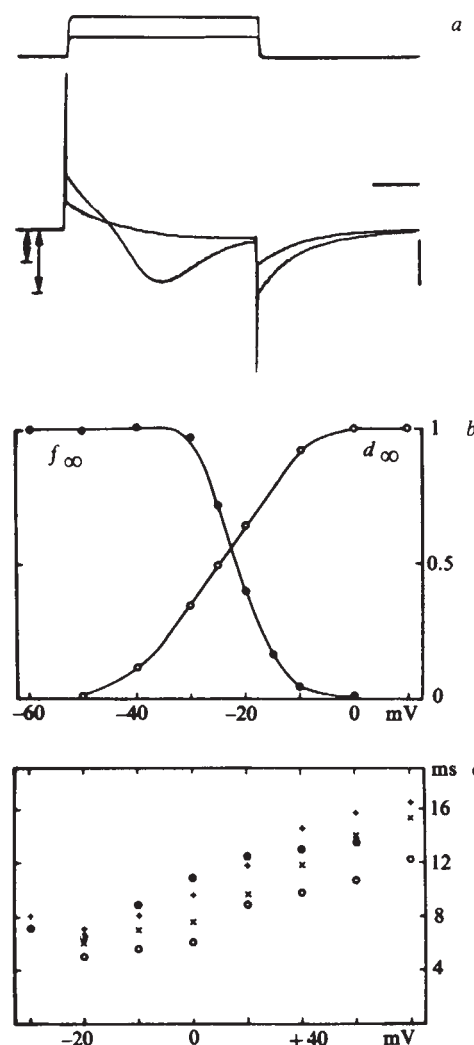


Fig. 5 (Right) Influence of the membrane potential on the conductance parameters d_∞ , f_∞ and τ_i . Activation was analysed at -50 mV; a short depolarising pulse (8 ms to -30 and -20 in *a*) activated the conductance to a degree which is proportional to the amplitude of the tail currents (arrows). Inactivation (not illustrated) was measured by imposing steps to +10 mV after 3-s pre-pulses to potentials between -70 and 0 mV. Having normalised the maximum effects to 1.0, the dimensionless variables d_∞ and f_∞ were plotted against the potential of the conditioning step (*b*). The inactivation time constant τ_i (*c*) was analysed according to Fig. 3d; the different symbols indicate four individual myocytes. Calibration marks indicate 4 ms and current from 0 to -1 nA.

Our data indicate that myocytes isolated from adult rat heart tissue can have electrical properties similar to those displayed by intact tissue. With regard to the slow inward calcium current, I_{si} , threshold, maximum amplitude and direction depended on membrane potential as in the intact tissue; the reversal potential is +55 mV, quite close to the values reported^{1,2}. Also, the influence of membrane potentials on the steady-state activation and inactivation curves is in good agreement with measurements on intact cardiac tissue from bovine or feline hearts^{1,2}. However, our τ_i values are similar to those estimated by us from currents recorded in rat heart ventricular trabeculae^{14,15}, that is, fast inactivation seems to be a peculiarity of the rat heart. This conclusion is supported by preliminary experiments with myocytes isolated from bovine heart tissue which revealed τ_i values of the order of 100 ms. We conclude that the glycocalyx, which is removed by the cell isolation procedure, is unlikely to be the structure originating or controlling the slow inward calcium current.

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Low intracellular pH and chemical agents slow inactivation gating in sodium channels of muscle

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Excitation of nerve or muscle requires an orderly opening and closing of molecular pores, the ionic channels, in the plasma membrane. During the action potential, Na channels are opened (activated) by the advancing wave of depolarisation, contributing a pulse of inward sodium current, and then are closed again (inactivated) by the continued depolarisation¹. As one approach both to obtaining molecular information on the Na channel and towards further defining the recently discovered kinetic interactions of the inactivation and activation gating steps^{2–4}, we have surveyed here the effects of chemical agents reported to slow or prevent Na channel inactivation. We find that many of the agents studied by others^{5–14} on invertebrate giant axons or vertebrate nerve act on our frog skeletal muscle preparation. In addition, we have discovered that simply lowering the intracellular pH nearly eliminates inactivation. The activation mechanism seems to resist modification.

Figures 1 and 2 show examples of a loss of inactivation gating following chemical treatments. The traces are families of sodium currents recorded from a cut piece of frog semitendinosus muscle fibre under voltage clamp by the Vaseline-gap technique¹⁵. The membrane was hyperpolarised for 56 ms and then depolarised to test potentials ranging from –90 to +70 mV in 16-mV intervals. In a normal muscle fibre bathed in Ringer's solution at pH 7 (Fig. 1a) or at pH 5 (Fig. 1e), the sodium currents have a transient time course, activating quickly and inactivating fully (at least 98%) during each 8-ms depolarising test pulse. However, after a 10-min exposure to 0.7 mM *N*-bromoacetamide (NBA) in the external medium (Fig. 1b), the inactivation process is modified, and sodium currents are still large at the end of 8 ms. Sodium channels close only after the membrane potential is stepped back to the holding potential, giving a brief inward sodium 'tail current'. Even with 75-ms test pulses, the currents in NBA-treated fibres fail to inactivate

completely (Fig. 2f) and the end of the pulse is followed by a prominent tail current (not faithfully recorded at the low sampling frequency used after the first 8 ms in the record shown). Similar modifications of inactivation gating are seen in a fibre treated for 36 min with internal iodate (Figs 1c, 2b) and in fibres treated for 38 min with 40 mM external formaldehyde (Fig. 2e) or for 43 min with 1.8 mM external glutaraldehyde (Fig. 2c) followed by another 6 min in 10 mM glutaraldehyde (Fig. 2d).

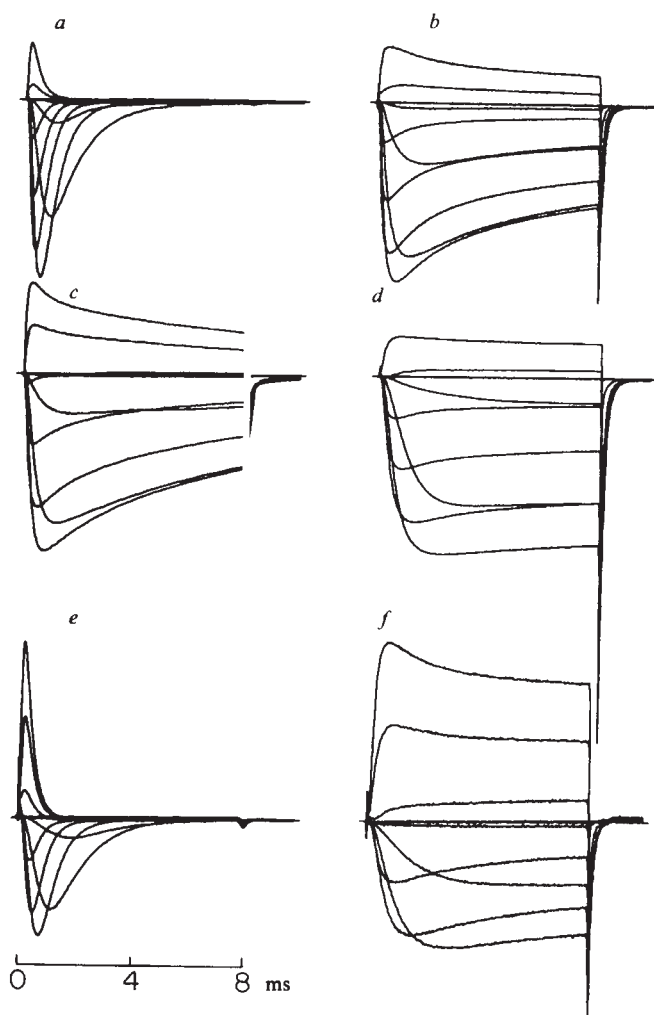
These observations with frog muscle parallel the reported effects on inactivation of 20–120 mM internal iodate in frog myelinated nerve^{5,6} and of 1 mM internal (but not external) NBA in squid giant axon⁷, and they are consistent with the action-potential-prolonging effect of 2 mM external glutaraldehyde in crayfish giant axons⁸. We also tested other agents reported to act on giant axons and obtained the results listed in the last two columns of Table 1. These agents seemed uniformly less promising for future use in our preparation, but most did slow the time course of inactivation.

In one series of experiments with fibres bathed in external solutions at pH 5, we found that inactivation remained normal when the buffer was 17 mM histidine (Fig. 1e) but became incomplete during 45 min when the pH 5 buffer was 28 mM acetate (Fig. 1f). This change could be slowly reversed, especially at an intermediate stage, by returning to low-pH histidine or to normal Ringer. On the hypothesis that acetic acid might cross the sarcolemma and act by acidifying the myoplasm, we tested the effect of lowering internal pH by placing 48–60 mM biphthalate buffer at one cut end of fibres bathed externally with flowing, well buffered solutions at neutral pH. Inactivation was nearly completely lost after 50 min with pH 4 buffer at one end and after 100 min with pH 5 buffer (Figs 1d, 2h). This treatment gave the most complete removal of inactivation of any we have tried and resulted in no loss of peak current. The effect could be further potentiated by external application of pH 5, acetate-buffered Ringer.

We also examined whether bisulphite ion, which has been reported to modify endplate channels¹⁶, also modifies gating in Na channels. When 25 mM bisulphite was applied for 100 min from inside or outside in a pH range of 6 to 9, there was no effect except a gradual reduction of current. However, if one cut end of the fibre was bathed for about 1 h in a pH 5.6 bisulphite solution and then the membrane was perfused externally with a pH 5.2 bisulphite Ringer's solution, inactivation was lost (Fig. 2g), and returned slowly after both bisulphite solutions had been washed away. We believe that external bisulphite acts here simply by accelerating the acidification of the myoplasm, perhaps by diffusion of the uncharged SO₂ species. Such synergism could be

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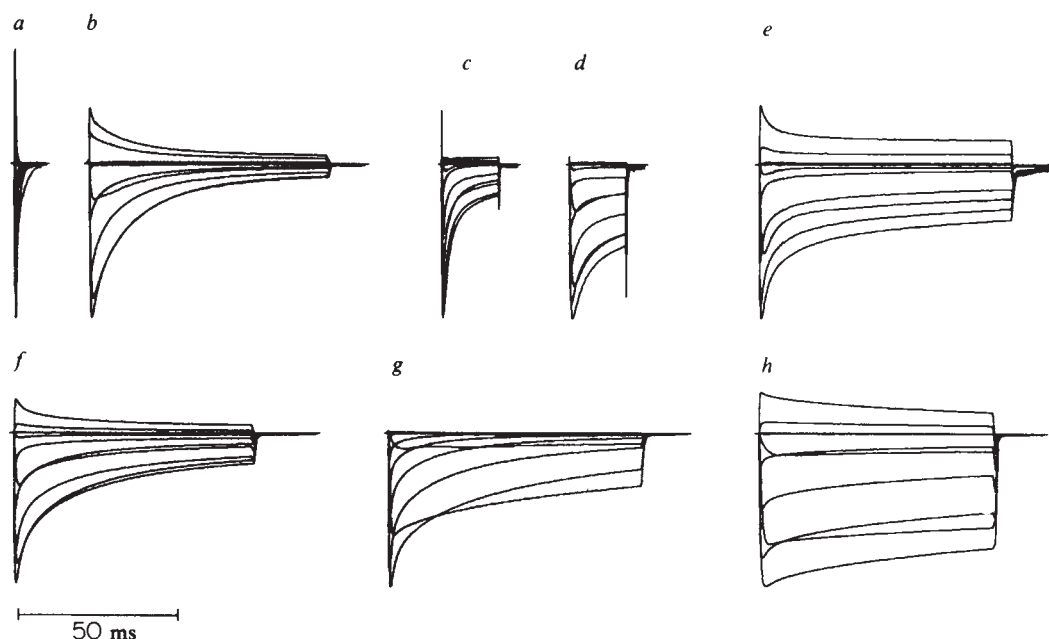
Fig. 1 Families of Na currents after treatments with agents that modify inactivation. Family *a* was recorded before, and family *b* at 8 min after, a 10-min exposure to 0.72 mM external NBA (in Ringer's buffered at pH 7 with 8 mM phosphate; internal solution: 60 mM KF, 60 mM CsF); fibre 6/19. Family *c* was obtained from fibre 6/20, at 14 min after the end of a 22-min internal application of iodate (60 mM NaIO₃, 60 mM CsF, then replaced by 20 mM Na phosphate buffer, pH 7, 100 mM CsF; external solution: Ringer's). The first 250 μ s of the tail current were not recorded. Records *d*, from fibre 6/6, were made at 103 min after the beginning of an internal treatment with pH 5, biphthalate buffer (48 mM K biphthalate, 24 mM NaOH, 60 mM CsF; outside: Ringer's). Family *e* was taken after 8 min in external Ringer's with 17 mM histidine buffer at pH 5; *f* was recorded later from the same fibre, 6/4/1, after a repeated, external application (total duration 42.5 min) of Ringer's with 48 mM acetate buffer at pH 5.1 (internal solution: 20 mM Na phosphate at pH 7, 100 mM CsF). Holding potentials -90 mV (*a-d*) or -74 mV (*e, f*); 8-ms depolarising pulses from -74 to +70 mV at intervals of 16 mV. Temperature 10-11 °C. Each record was scaled for the same amplitude of the largest inward (*a-d*) or outward (*e, f*) current; amplitudes were (*a-f*): 2.69, 0.93, 2.57, 4.64, 1.02, 0.272 μ A. The largest control currents for *c* and *d* were 3.86 and 5.55 μ A.



expected from all agents that can permeate to the myoplasm and there, by dissociation or other chemical reactions, generate free protons. In the test tube we found that added NBA, formaldehyde, glutaraldehyde or bisulphite lowers the pH of a histidine buffer solution within a few minutes from pH 7 to values lower than 4 by chemical reactions. We therefore cannot exclude the possibility that a slowly developing reduction of intracellular pH also contributes to the efficacy of these agents in muscle fibres.

Where do the chemical modifiers act? Two of the treatments we tried, iodate and low pH, were effective only from the inside of the membrane. External iodate up to 120 mM was totally without effect and low external pH gave only the known small effects¹⁷ seen in Fig. 1*e*. Other small, uncharged agents such as NBA and aldehydes were effective when applied outside, but as they can readily diffuse across membranes, they might also be acting inside. The chemical specificity of the agents we have tried has been discussed extensively elsewhere, and other

Fig. 2 Slowed Na inactivation seen with long step depolarisations. *a*, Records from a normal fibre, 6/20 (Ringer's outside; 60 mM NaF, 60 mM CsF inside). *b* is the same fibre 16 min after the end of a 22-min internal exposure to iodate (same fibre as Fig. 1*c*). *c* is taken from fibre 4/26 after 43 min external treatment with glutaraldehyde (1.8 mM in Ringer's; inside: 120 mM CsF), and *d* from the same fibre after a further 6 min exposure to 10 mM glutaraldehyde in Ringer's. Records *e* were taken from fibre 4/25 after 38 min external application of 40 mM formaldehyde (with 0.04% methanol) in Ringer's (inside: 120 mM CsF). Family *f* was recorded



17 min after the end of a 10-min external exposure to 0.72 mM NBA (same fibre as in Fig. 1*a, b*). *g* is from fibre 4/20, which was treated for 72 min with internal bisulphite (19 mM NaHSO₃ in 120 mM CsF, pH 5.6), simultaneously exposed for 5 min (at 46-51 min) to external bisulphite (30 mM NaHSO₃) in Ringer's, pH 5.2, and then washed for 21 min with Ringer's. Records *h* were taken after 110 min of internal exposure to pH 5 biphthalate buffer (same fibre as in Fig. 1*d*). Same depolarisations as in Fig. 1. Temperature 10-11 °C. Currents were scaled to the same size; the maximum inward currents were (*a-h*): 3.85, 2.32, 0.51, 0.29, 0.63, 0.89, 1.99, 4.63 μ A; corresponding control currents were (*c-h*): 0.99, 0.99, 1.70, 2.69, 1.77, 4.64 μ A.

Table 1 Other experiments on frog muscle with agents reported to modify Na inactivation

Agent	Ref.	Modes of application	Our experiments	
			Depression of Na current*	Action on inactivation†
<i>N</i> -Bromosuccinimide (NBS)	7, 9	External, 1–2 mM	+	++
2, 4, 6-Trinitrobenzene sulphonic acid (TNBS)	10	External, 3–30 mM Internal, 60–120 mM (pH 7 or 9)	+ ++	– +
4-Acetamido-4-isothiocyano-stilbene-2,2'-disulphonic acid (SITS)	10	External, 1–4.4 mM Internal, 11.5 mM	+ ++	– +
Isethionylacetamide hydrochloride (IAH)	10	External, 3–10 mM Internal, 10–70 mM	– –	– –
Glyoxal	11	External, 6–60 mM Internal, 11–60 mM	– –	– +
Tannic acid	8	External, 2.2 mM Internal, 0.3 mM	++ –	– –
Picric acid (TNP)	13	External, 2.2 mM	++	+
High internal pH	14	Internal, pH 9.5–10	++	–

* Current depressed by less (+) or more (++) than one half.

† Inactivation slowed, but almost complete after 8 ms (+), or markedly incomplete after 8 ms (++).

authors^{7,10,11,14,18} have argued that modifications at tyrosine and arginine and reaction with lysyl- or N-terminal amino groups on the axoplasmic side of the membrane all remove Na inactivation.

Although inactivation gating was strongly modified by chemical treatments, activation gating seemed hardly affected. Figure 3 shows the voltage dependence of peak Na permeability and of the half-rise and half-fall times of the Na current during the test pulse. The normalised permeability-voltage curves are quite similar before (solid line) and after (dashed line) the chemical treatments except that with NBA, iodate and biphthalate (Fig. 3a, c, d) there is a small (<8 mV) shift to the left along the voltage axis. The half-rise times (triangles) are similarly

unchanged with NBA, formaldehyde and iodate (Fig. 3a–c), but are longer with the low internal pH (biphthalate, Fig. 3d). As is already clear, the fall times (circles) are greatly increased after all treatments, with low internal pH being the most potent.

These experiments confirm that inactivation and activation gating processes may be dissociated functionally, as if they are subserved by different parts of the channel. Perhaps the activation machinery is physically more deeply buried in the membrane and therefore less susceptible to chemical modification¹⁹. However, it must be remembered that all treatments except low internal pH also reduced the peak Na currents, and in some of these cases the reduction might reflect alterations in parts of the channel associated with activation.

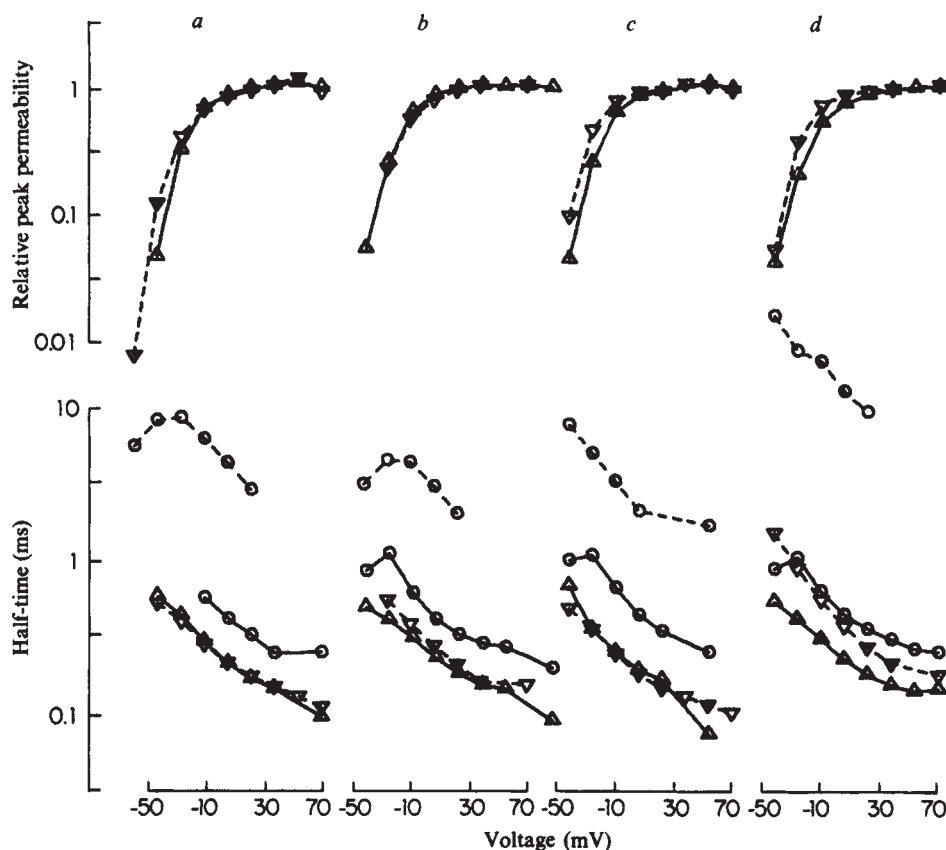


Fig. 3 Effects of agents on parameters of Na current. Upper: peak permeability, calculated from the Goldman flux equation²⁰; lower: half-times for rise and fall of Na current. Δ , Time to rise to half of peak value; $\circ$, time after peak to fall half-way to value at 75 ms. Controls are connected by solid lines and modified parameters by dashed lines; abscissa is the voltage of the test pulse. *a*, Before and after treatment with 0.72 mM external NBA (fibre 6/19, see Figs 1a, b, 2f). *b*, Before and during treatment with 40 mM external formaldehyde (fibre 4/25, see Fig. 2e). *c*, Before and after treatment with 60 mM internal iodate (fibre 6/20; Figs 1c, 2a, b). *d*, Before and during internal exposure to pH 5, biphthalate buffer (fibre 6/6; Figs 1d, 2h). Temperature 10–11 °C.

The chemical separability does not, however, exclude the possibility of interactions between the gating processes in normal channels: activation gates may still operate differently in inactivated and non-inactivated channels²⁻⁴.

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Distribution of membrane anionic sites on B16 melanoma variants with differing lung colonising potential

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The interaction of metastatic cells with their environment is mediated to a large extent by the cell surface¹⁻⁵. Although several biochemical differences between tumour cells with low or high metastatic potentials have been reported⁶⁻¹⁰, the specific surface characteristics associated with metastasis have not yet been identified. One distinctive feature of murine B16 melanoma variants with low (B16-F1, B16-F10^{Lr}) or high (B16-F10) lung colonisation potentials^{11,12} is their propensity to aggregate *in vitro* with other tumour cells (homotypic clumping)^{13,14}, or with host cells (heterotypic clumping)^{15,16}. The initial sites for membrane-membrane recognition, contact and subsequent interaction are thought to be associated with dense membrane anionic sites¹⁷⁻²⁰. In the experiments reported here we determined that the distribution of cell-surface dense anionic sites, examined ultrastructurally with the use of cationised ferritin (CF), is correlated with tumour cell aggregation *in vitro* and/or production of pulmonary tumour colonies following intravenous (i.v.) injection into syngeneic recipients.

The B16-F1 (parent, intermediate metastatic potential), B16-F10 (selected *in vivo*, high metastatic potential)¹¹ and B16-F10^{Lr} (selected *in vitro* for resistance to lysis by syngeneic lymphocytes, low metastatic potential)¹² were maintained *in vitro* as described previously¹². Cells from semiconfluent monolayer cultures were collected with a short (2 min) treatment of 2 mM EDTA in Ca²⁺- and Mg²⁺-free phosphate buffer saline (PBS), pH 7.2. The cells were washed in PBS and pipetted gently to dissociate any cell clumps. In all experiments, only cell suspensions of >90% viability, as measured by trypan blue exclusion, that were free of cell aggregates were used for *in vivo* or *in vitro* studies.

The biological behaviour *in vivo* of the cell lines was evaluated by i.v. injection of 5 × 10⁴ viable cells into groups of 10 mice

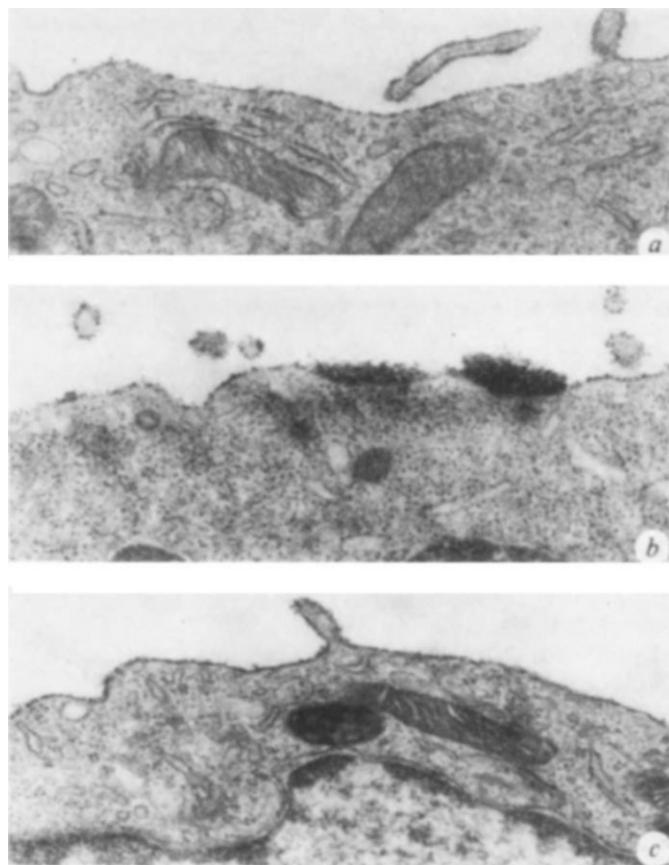


Fig. 1 Distribution of cationised ferritin on the cell surface of B16 melanoma cell lines: a, B16-F1; b, B16-F10; and c, B16-F10^{Lr}. Cell suspensions were fixed with 2.5% glutaraldehyde in Na-Veronal buffer (pH 7.4) for 60 min at 24 °C. Following washing three times with the same buffer the fixed cells were incubated for 3 min with 0.33 mg ml⁻¹ cationised ferritin (Miles Yeda)¹⁴. The unbound cationised ferritin was washed away and the cells were post-fixed with 1% buffered OsO₄ for 60 min at 24 °C and stained *en bloc* with a solution of 1% uranyl acetate. The cells were dehydrated with a graded series of ethanol followed by propylene oxide and embedded in Emix resin (Ted Pella, Inc.). Thin sections were obtained with an LKB ultramicrotome and analysed with the aid of Hitachi HU-12A electron microscope at an operating voltage of 75 kV. Serial sections of 600 Å through the CF clusters established that the observation was not due to an artefact of sectioning.

Table 1 Distribution of cationised ferritin clusters on surface of B16 melanoma cells

Cell lines	No. of cells with CF clusters/ no. of cells examined	Per cent of total
F1	38/180	21
F10	114/279	41
F10 ^{Lr}	51/180	28
F10*	27/122	22

Methods as described in Fig. 1. The results represent the average obtained from a random cell thin sections, and blind scoring. The experiments were repeated twice with cells grown from different (frozen) stock cultures with similar results.

* B16-F10 cell suspensions (1 × 10⁶ cells ml⁻¹) in 5% FCS in medium were placed in a 15-ml conical tube and rotated gently for 60 min at 37 °C. The aggregates were then allowed to sediment for 30 min at 24 °C. The upper third layer of the suspension was collected, washed with PBS, fixed and stained with CF.

Table 2 Homotypic aggregation of B16 melanoma cell lines

Cell line	Per cent cells in aggregates
F1	32
F10	52
F10 ^{Lr}	11
F10*	25

B16 melanoma cell suspension (1×10^6 cell ml⁻¹) in medium containing 5% FCS were placed into a 15-ml conical tube and rotated gently for 60 min at 37 °C. An aliquot from each suspension was placed into a haemocytometer and the number of single cells per field was determined. The percentage of aggregates was calculated from the cell number in control experiments where the cells were incubated at 4 °C.

* B16-F10 cell suspension was incubated for 30 min with 0.5 µm ml⁻¹ of neuraminidase, then washed and allowed to aggregate as above.

each. The mice were killed 21 days later and the number of tumour colonies in the lungs was determined. The median number of metastases produced by B16-F1, B16-F10 and B16-F10^{Lr} cells was 12, 130 and 2, respectively. These results agree with earlier reports^{6-8,11,12}. Quantitative determination of both intracellular and extracellular sialic acid²¹, demonstrated no differences among the three B16 lines as each line contained about 0.025 µmol of sialic acid per 10⁶ cells.

The distribution (topography) of the cell-surface anionic sites was examined by electron microscopy of cells treated with CF as described previously²². To avoid a possible artefact of clustering of the ligand on live cells²³, we examined the distribution of CF on glutaraldehyde prefixed cells. Striking differences in ferritin binding on the surface of the B16-F10 (high metastasis) and B16-F1 and B16-F10^{Lr} (low metastasis) were observed (Fig. 1, Table 1). Specifically, in over 70% of the B16-F1 and B16-F10^{Lr} cells the CF was bound to the cell surface as a continuous discrete layer where each CF particle was separated from an adjacent particle by ~100 Å. In contrast, in 41% of the B16-F10 cells the ferritin particles were clustered on the plasma membrane (Table 1). In only 21% of B16-F1 and 28% of B16-F10^{Lr} cells, were small clusters of CF particles found.

The frequency at which the cells in the population demonstrated dense cell-surface anionic sites correlates with the degree of *in vitro* homotypic aggregation (Table 2). In this system at least, homotypic aggregation *in vitro* is energy and fetal calf serum dependent, as at low temperatures (4 °C) or in absence of serum no significant aggregation of B16 cells could be detected. Neuraminidase treatment (0.5 units ml⁻¹, 30 min at 37 °C) of B16-F10 melanoma cells before the homotypic aggregation assay reduced the number of cells per aggregate (Table 2).

Not all cells in the B16-F10 suspension participate in the process of homotypic aggregation. The frequency of dense anionic sites among single (unaggregated) B16-F10 cells was

Table 3 Incidence of pulmonary metastases in C57BL/6 mice following the i.v. injection of B16-F10 melanoma cells

Source of B16-F10 cells	Median (range)* of pulmonary metastases
1. Control, total population†	63.5 (37-96)
2. Single cells recovered from aggregates‡	66 (14-146)
3. Single cells recovered from supernate§	29 (1-100), P < 0.001

* Ten mice per group were injected with 5×10^4 viable cells (>90%) in 0.2 ml of PBS. Pulmonary metastases were counted with a dissecting microscope 18 d after i.v. injection.

† B16-F10 cells were incubated in CMEM and 5% FCS for 60 min at 4 °C and then washed with PBS.

‡ B16-F10 aggregates (Table 2) were sedimented and dissociated by gentle pipetting. The cells were washed in PBS.

§ Single cells obtained from upper third layer on the suspension (unaggregated cells).

|| Statistically significant group 3 versus 2 or 1.

remarkably similar (22%) to that observed in the B16-F1 and B16-F10^{Lr} suspensions. Evaluations of CF clusters on the surfaces of the cells within the aggregate could not be accomplished as the CF molecules did not penetrate the aggregates. In the final experiments we injected syngeneic mice i.v. with single cell suspensions consisting of B16-F10 cells (viability of >90%) from: the starting (unaggregated) population; those recovered from clumps; or those that did not participate in the aggregation process. The data of a representative experiment demonstrate that B16-F10 cells that did not participate in the homotypic aggregation process *in vitro* formed fewer pulmonary lung colonies than did single B16-F10 cells recovered from cell aggregates (Table 3).

In conclusion, we found no quantitative differences in total cellular sialic acid among three cell lines of the B16 melanoma with different metastatic propensities. In contrast, differences in the topography and display of dense anionic sites were detected among the lines. The visualisation of cell-surface dense anionic sites may be a useful tool for the investigation of subtle changes in the cell surface of metastatic cells. Such changes might account for the differences in the propensity of the B16 melanoma cells to form experimental pulmonary tumour colonies.

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Peanut lectin binding properties of germinal centres of mouse lymphoid tissue

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Peanut (*Arachis hypogaea*) lectin (PNL) has been shown to agglutinate the 90% of cells from murine thymus which are supposed to be immature cortical thymocytes¹. Further studies on the numbers of thymocytes binding fluorescein isothiocyanate conjugated PNL (FITC-PNL) confirmed the large proportion of PNL binding cells^{2,3}. In other organs such as bone marrow, spleen and peripheral lymph nodes, smaller proportions of PNL positive cells have been recorded^{2,3}. PNL-positive cells outside the thymus have been reported to be either Thy 1-positive or null cells^{2,3}. It has also been suggested that PNL

binding may be a marker for immaturity not only in relation to T lymphocytes² but also amongst haematopoietic stem cells⁴. Thus PNL binding as an aspect of lymphocyte differentiation is a matter of considerable interest. The current study describes the distribution of horseradish peroxidase-conjugated PNL (HRP-PNL) on frozen sections of mouse lymphoid organs. It seems that PNL binds to cells in germinal centres but not to those in some other areas containing activated lymphocytes. There is good correlation between the presence of PNL-binding germinal centres in frozen sections of lymphoid organs and the number of PNL-binding cells counted in cell suspensions from the same organs.

In 8–12-week-old male CBA mice the proportions of lymphocytes which were able to bind FITC-PNL in cell suspensions from thymus, spleen, peripheral lymph nodes and bone marrow (Table 1) were found to be similar to those previously reported^{2,3}. However, the observation that 20% or more of Peyer's patch lymphocytes bind PNL led to the investigation of the localisation of the PNL-binding cells in Peyer's patches using frozen sections stained with HRP-PNL (Fig. 1a). It can be seen that there is a close correlation between the PNL-binding area and the germinal centre which can be observed in sections conventionally stained with haematoxylin and eosin (H&E) (Fig. 1b). Comparable results were obtained using FITC-PNL or H&E on frozen sections of rat Peyer's patches. Peripheral lymph nodes which in unstimulated laboratory mice usually lack germinal centres, did not stain with HRP-PNL but the few germinal centres to be found in spleen from unstimulated mice did prove to be PNL-positive. So far all germinal centres have bound PNL but it should be stressed that much more material must be examined before any great weight can be put on this finding. PNL binds to terminal galactosyl residues with the highest affinity for the sequence β -D-galactose-(1 $\rightarrow$ 3)D-N-acetylgalactosamine⁹. The binding of PNL to germinal centres was completely abrogated in the presence of 0.1M galactose but not 0.1M glucose or mannose. Thus the binding of PNL to germinal centre cells is probably to terminal galactose residues on the cell surface.

Germinal centres contain dividing cells and their lymphocytes are pyronin-positive, indicating a higher degree of metabolic activity than is present in resting lymphocytes¹⁰. It was therefore of interest to determine whether PNL would bind to other activated lymphocyte populations. Local stimulation with the

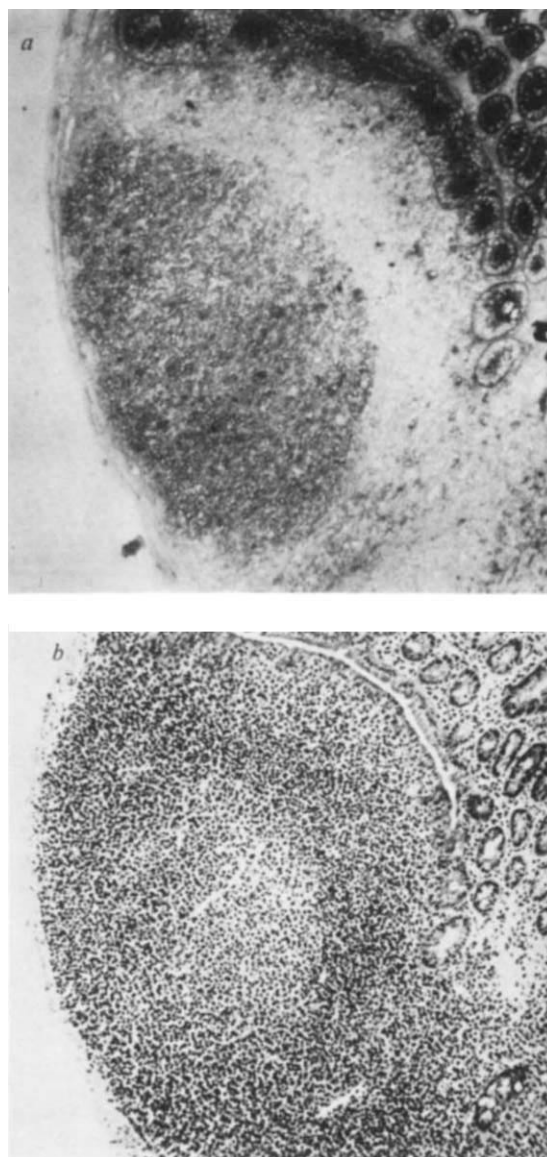


Fig. 1 Binding of HRP-PNL on frozen sections of murine Peyer's patches (a) and section from the same block conventionally stained with H & E (b). Peanut lectin was prepared according to Table 1 legend. HRP-PNL conjugate was prepared according to the method of Nakane⁸. Frozen sections (4- μ m thick) of mouse Peyer's patch were air dried, and stained with HRP-PNL (20 μ g PNL per ml) for 30 min at room temperature. Sections were washed three times in PBS and staining was developed using 3, 4, 3', 4'-tetra-aminobiphenyl hydrochloride. Sections were washed again in PBS, dehydrated and mounted. Magnification $\times 105$.

Table 1 Percentage of FITC-PNL binding cells in lymphoid organs of unstimulated CBA mice

Expt no.	1	2	3
Peripheral lymph node	4.0	3.0	
Spleen	6.0	5.0	
Thymus	76.0	82.7	88.0
Bone marrow	4.0		
Peyer's patch	26.7	26.0	19.4

Peanut lectin was prepared according to the method of Lotan *et al.*⁵, with the major exception that the final affinity purification step was performed using a column of lactose bound to epoxy activated Sepharose 6B from which the lectin was displaced by 50 mM galactose. The preparation possessed haemagglutinating activity of 31,000 units per mg towards neuraminidase-treated human erythrocytes, and showed only one protein band on disc electrophoresis at pH 8.6. PNL was conjugated to FITC⁶ in the presence of galactose to produce a FITC/PNL molar ratio of 2.7. Cell suspensions were prepared as previously described⁷ and 100 μ l of cells at 10^7 per ml in PBS containing 0.05% sodium azide were mixed with 100 μ l FITC-PNL (50 μ g PNL per ml) in phosphate-buffered saline (PBS) containing azide. After 30 min incubation at 37°, the cells were washed three times and resuspended in 100 μ l of PBS containing azide. Cells were examined under fluorescence optics using a Zeiss epifluorescence condenser IV/F on standard 18 microscope. Only viable cells (distinguishable from dead cells under phase contrast) with strong membrane fluorescence were scored as positive. In each experiment (done on different days) the relevant organs from at least four mice were pooled, tubes were counted in duplicate and the mean is shown. 200 cells per tube were scored.

contact sensitising agent, oxazolone, results in intense T-cell proliferation in the paracortex of the draining lymph nodes at 3 days¹¹. Frozen sections of such nodes did not bind PNL to an enhanced degree nor was it possible to detect an increase in the numbers of PNL-binding cells in cell suspensions from lymph nodes activated in such a manner (Table 2). However, at later times (days 7–15) increased numbers of PNL-binding cells were observed (Table 2). This increase was correlated with the time of appearance of germinal centres. No increase in the numbers of PNL-binding cells was found in spleen cells which had been activated *in vitro* with either lipopolysaccharide or with phytohaemagglutinin (Table 3). Thus PNL does not bind to activated lymphocytes *per se* but only to cells in particular anatomical locations. The fact that PNL-binding cells can be recovered from unfixed cell suspensions and demonstrated by agglutination¹ suggests that PNL is membrane binding.

Table 2 Percentage of FITC-PNL binding cells in the axillary lymph nodes of mice stimulated with oxazolone

	Days after oxazolone	No. of mice	Mean
Expt 1	0	3	4.0 ± 0.6
	3	4	3.4 ± 1.5
	7	2	12.3 ± 1.0
	13	1	13.9
	17	1	7.4
Expt 2	0	4	2.8 ± 1.2
	4	4	3.3 ± 0.9
	11	4	15.3 ± 3.4
	15	4	19.2 ± 6.0

PNL and FITC-PNL were prepared as described in the legend to Table 1. Mice were painted on the shaved flanks with 3 mg of 4-ethoxymethylene-2-phenyl-oxazolone (oxazolone, BDH) dissolved in 100 µl of ethanol. At various times after immunisation, mice were killed and cell suspensions from the draining axillary lymph nodes were prepared, they were washed twice and resuspended at a concentration of 10^7 per ml in PBS containing 0.05% sodium azide. The number of FITC-PNL binding cells was estimated as described in Table 1. Results are the mean ± s.d. from individual mice.

Table 3 Percentage of FITC-PNL binding cells in spleen cultured with LPS or PHA

	Time (h)		
	24	48	72
LPS	4.9 ± 1.6	4.2 ± 1.3	2.8 ± 1.0
PHA	4.9 ± 0.8	5.3 ± 3.4	4.8 ± 3.0

Spleen cells were cultured in RPMI1640 containing 10% fetal calf serum, 300 mM glutamine, 200 unit ml⁻¹ penicillin and 150 µg ml⁻¹ streptomycin, in 1 ml cultures at a concentration of 2×10^6 cells per ml with phytohaemagglutinin (PHA, Wellcome, 2.5 µg ml⁻¹) or 4×10^6 cells per ml with lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Difco, 50 µg ml⁻¹). After culturing (at 37°C in an atmosphere of 7% CO₂, 10% O₂, 83% N₂) for 24, 48 or 72 h, cells were collected and the numbers of FITC-PNL binding cells were estimated as described in Table 1. Results are the mean ± s.d. from four replicate cultures. Control cell suspensions of uncultured spleen revealed 6.0 ± 2.0, 5.5 ± 1.7 and 4.7 ± 1.1% FITC-PNL binding cells at 24, 48 and 72 h respectively. A control thymocyte suspension done at 72 h revealed 82% PNL binding cells.

The origin of cells in germinal centres has not been fully determined. Weissman¹² has shown that there are some T cells present by the use of fluoresceinated anti-T cell antiserum. However, neither the numbers nor the pattern of dispersion of these cells corresponds to that seen with PNL. Thus the PNL-positive cells are unlikely to be mainly T lymphocytes. In the same study Weissman demonstrated that a substantial proportion of the area of germinal centres was immunoglobulin positive. This finding must be viewed with some caution before it is assumed that many or all of the cells in germinal centres are surface immunoglobulin-positive B lymphocytes. It is known that, in at least some germinal centres, there are dendritic reticular cells which bind antigen-antibody complexes and which have cell membranes with vast processes on which the complexes are to be found¹³. Preliminary studies in this laboratory with double staining techniques on cell suspensions from Peyer's patches have shown that some of the PNL-positive cells appear to be surface immunoglobulin positive, whereas others are not obviously so. Thus the anatomic origins of PNL-positive germinal centre cells are not clear, although it seems likely that some or all of them are B lymphocytes.

It is an intriguing coincidence that cortical thymocytes and germinal centre cells bind PNL. These observations, together with Reisner's suggestion⁴ that PNL binding is a marker of immaturity among T cells and cells of the haematopoietic stem cell population, again raise the possibility that germinal centres harbour a population of immature B cells^{14,15}. Use of PNL to separate germinal centre cells should allow their properties and functions to be characterised further.

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Protective monoclonal antibodies recognising stage-specific merozoite antigens of a rodent malaria parasite

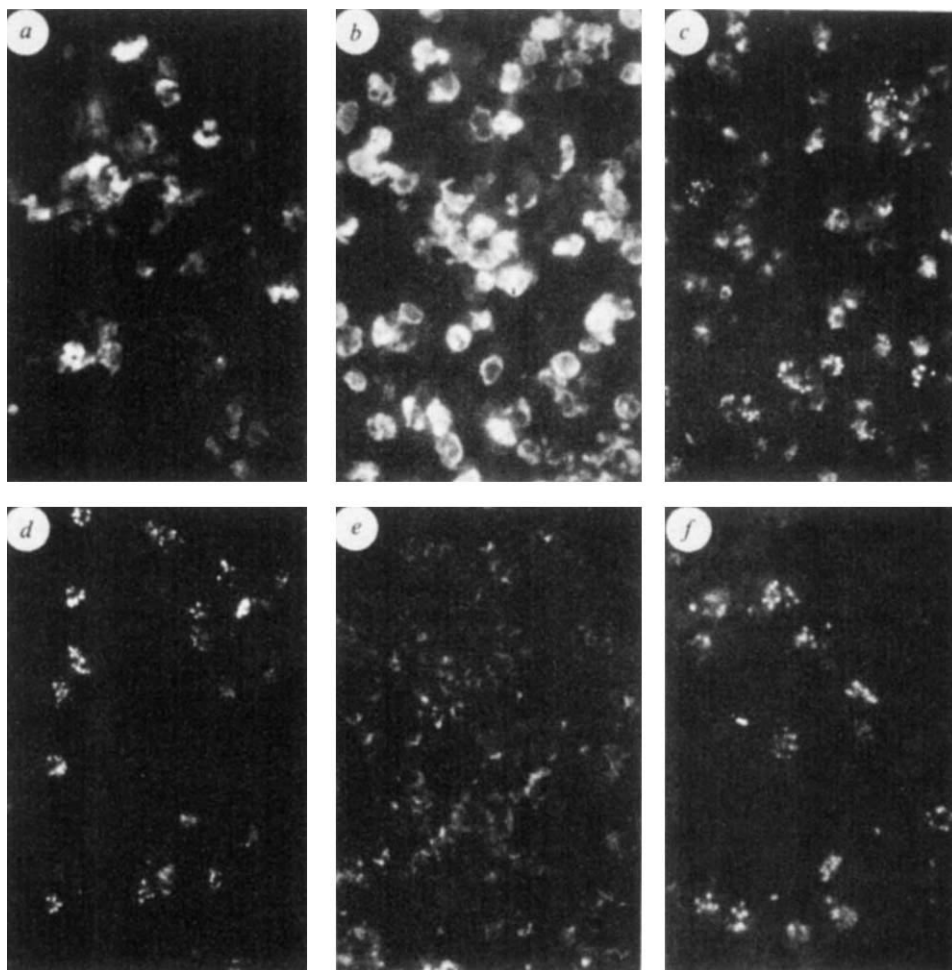
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Immunity to malaria is mediated, at least in part, by antibody. Resistance to infection has been passively transferred with immune serum or its immunoglobulin fraction in human¹, simian² and rodent^{3,4} malaria. However, because of the structural and antigenic complexity of the malaria parasites^{5,6}, it has proved difficult to identify and characterise those parasite antigens against which protective antibody is directed. We have produced several hybrid cell lines secreting monoclonal antibodies against the rodent malaria parasite, *Plasmodium yoelii*, and we now report that, of the antibodies tested, only those specific for antigens exclusive to the merozoite were protective in passive transfer experiments. Other anti-*P. yoelii* monoclonal antibodies, apparently recognising antigens in the membrane of the infected erythrocyte, were not protective on passive transfer. The protective monoclonal antibodies should be useful in the isolation of the important antigens of this parasite.

Spleen cells from two *P. yoelii*-immune BALB/c mice were fused with P3-NS1/1-Ag4-1 myeloma cells in the presence of polyethylene glycol⁷. The cells were dispensed into 144 tissue culture wells in 2-ml volumes of hypoxanthine-aminopterin-thymidine-selective medium⁸, using RPMI-1640 medium supplemented with 10% fetal calf serum as a base⁹. After 10 days, the culture supernatants were tested for *P. yoelii*-specific antibody by indirect immunofluorescence (IIF). Of 143 cultures tested, 38 were positive, 20 producing antibody apparently directed against the infected erythrocyte membrane and 18 producing antibody against the parasite itself. From the initial screening, five distinct IIF specificities were detected. Cultures representative of each of the IIF specificities were expanded in selective medium without aminopterin, and hybridoma lines were subsequently cloned in semi-solid agar overlaid with medium.

Fig. 1 IIF staining reactions produced by: *a*, whole anti-*P. yoelii* immune serum diluted 1 in 1,000, and *b-f*, 1 in 10,000 dilutions of sera from mice carrying ascites tumours derived from cloned hybridoma cell lines: *b*, WIC 25.1; *c*, WIC 25.23; *d*, WIC 25.37; *e*, WIC 25.54; *f*, WIC 25.77. Supernatants from cultures of these lines produced IIF reactions identical to those produced by the sera. To produce monoclonal antibodies, 2×10^8 spleen cells from BALB/c mice recovered from mild *P. yoelii* 17X infection and rechallenged three times with virulent *P. yoelii* (YM strain) were mixed for fusion with 2×10^7 P3-NS1/1-Ag4-1 myeloma cells⁹. To prepare antigen for the IIF test, BALB/c mice were infected with virulent *P. yoelii* 24 h before subcutaneous injection with cyclophosphamide (200 mg per kg). After 4 days, when parasitaemia was >60%, infected blood was enriched for schizonts by centrifugation through isopaque/Ficoll¹² (2,000g for 20 min at 20 °C). The enriched upper layer of cells was washed in phosphate-buffered saline (PBS), diluted 1 in 100 in PBS, and applied dropwise to glass slides. The antigen preparation was allowed to dry at room temperature, then slides were stored at -20 °C. Giemsa staining of preparations showed that all red cells were parasitised (30% schizonts, 70% trophozoites approximately). Free merozoites were also observed. The procedure for the IIF test has been described elsewhere¹³; in the present study, background fluorescence was minimised by using immunoabsorbant-purified rabbit anti-mouse Ig. ($\times 180$).



The five cloned lines secreted antibodies covering the range of IIF specificities originally detected. Whereas polyspecific serum from immune mice produced a generalised staining of *P. yoelii*-infected erythrocytes in the IIF assay, the monoclonal antibody specificities produced characteristic, restricted patterns of fluorescence (Fig. 1). Thus, with specificity 25.1, the membranes of a subpopulation of infected erythrocytes fluoresced, whereas specificity 25.54 bound to the membranes of all infected erythrocytes with a distinct patchy staining pattern. Specificity 25.23 bound to an antigen common to all developmental stages of the blood form of the parasite. Specificities 25.37 and 25.77 bound to antigens only present in merozoites, free and within mature schizonts. All merozoites in the antigen preparation apparently reacted with the two merozoite-specific antibodies, but the

possibility that a minor subpopulation of merozoites failed to react could not be excluded.

The antibody products of the five lines were characterised with respect to immunoglobulin subclass by double diffusion using subclass-specific antisera. All were found to be of the IgG class (Table 1). They were also screened for cross-reaction against uninfected BALB/c erythrocytes and against a heterologous rodent malaria parasite, *Plasmodium vinckei petteri*, by IIF. None reacted with the uninfected erythrocytes but all except the merozoite-specific antibody secreted by line WIC 25.37 cross-reacted with *P.v.petteri* antigens (Table 1).

Serum pools containing high titres of anti-*P. yoelii* monoclonal antibodies were obtained from hybridoma tumour-bearing BALB/c mice (Table 2). These sera were tested in passive transfer experiments for their effect on the course of virulent *P. yoelii* infection (Fig. 2). Mice receiving monoclonal antibody specificities 25.1 and 25.54, apparently directed against infected erythrocytes, were not protected against *P. yoelii*. Neither was protection transferred with serum containing a high titre of specificity 25.23, directed against an antigen common to all developmental stages of the asexual erythrocytic parasite (trophozoites, schizonts and merozoites). However, transfer of sera containing the antibodies specific for late-appearing merozoite antigens, specificities 25.37 and 25.77, resulted in inhibition of *P. yoelii* parasitaemia, and none of these mice died. Whole immune serum was even more effective, causing the elimination of patent parasitaemia within 48 h of transfer. The protective activity of the monoclonal antibodies was not restricted to one IgG subclass, as specificity 25.37 was an IgG1 and specificity 25.77 was an IgG2a.

The eventual recovery of those groups treated with monoclonal anti-merozoite antibody was probably not mediated by the transferred serum, but rather by the recipients' immune

Table 1 Characteristics of the anti-*P. yoelii* monoclonal antibodies secreted by five cloned hybridoma lines

Hybridoma line	IIF specificity of secreted antibody	IIF cross-reactivity against <i>P.v.petteri</i>	Immunoglobulin subclass
WIC 25.1	Infected erythrocytes	+	IgG2a
WIC 25.23	Trophozoites, schizonts and merozoites	+	IgG2a
WIC 25.37	Merozoites	-	IgG1
WIC 25.54	Infected erythrocytes	+	IgG3
WIC 25.77	Merozoites	+	IgG2a

IIF specificity was determined by testing against *P. yoelii*, YM strain. For immunoglobulin subclass determination, concentrated culture supernatant samples were tested by double diffusion in agar against rabbit antisera specific for the mouse immunoglobulin subclasses IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA.

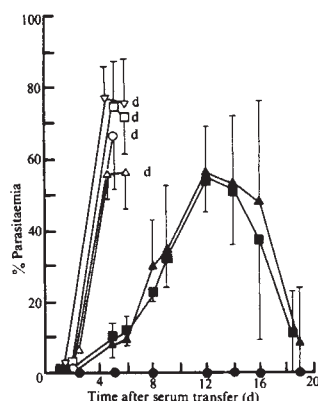


Fig. 2 The course of *P. yoelii* infection in groups of five BALB/c mice injected intraperitoneally with 0.5 ml of normal mouse serum (○), *P. yoelii*-hyperimmune serum (●), or serum from mice carrying tumours derived from cloned hybridoma lines: WIC 25.1 (△), WIC 25.23 (□), WIC 25.37 (▲), WIC 25.54 (▽) and WIC 25.77 (■). Serum was transferred when parasitaemia had reached 0.01–0.1%. Mean survival times are indicated (d). The vertical bars indicate standard deviations.

response. The direct effect of the transferred antibody was seen during the week following transfer, when the normally fulminating *P. yoelii* YM infection was retarded, following a course more typical of self-limiting *P. yoelii* 17X infection.

Inhibition of red cell invasion by merozoites may involve the blocking by antibody of receptor determinants on the merozoite surface. A polyspecific antiserum would be expected to be more efficient in receptor blocking than a monospecific antiserum. This may explain the greater degree of protection transferred with whole hyperimmune serum than with serum containing a single anti-merozoite specificity. An interesting alternative possibility is that the delayed parasitaemia observed in mice treated with specificities 25.37 and 25.77 was due to the emergence of a population of merozoites not reacting with these antibodies.

It is not known whether monoclonal specificities 25.37 and 25.77 recognise distinct merozoite antigens or different determinants on the same antigen. They give similar IIF staining reactions against *P. yoelii* (Fig. 1), but the observation that specificity 25.77 is cross-reactive with *P. v. petteri* indicates that this antibody possesses a binding specificity distinct from that of the other protective monoclonal antibody species. Using monoclonal antibodies and immunoprecipitation, we are now in the process of identifying the parasite antigens involved.

Our findings do not support the view that antibodies specific for parasite antigens present in the membrane of the infected erythrocyte are protective¹⁰, although they do indicate the presence of at least one erythrocyte membrane-localised *P. yoelii* antigen, against which a substantial but apparently non-protective antibody response is made in the immune host. However, we have tested only two membrane-specific monoclonal antibodies, and our results do not rule out the possibility

that other membrane-specific antibodies may prove protective. Furthermore, although specificities 25.1 and 25.54 bound to the membranes of infected erythrocytes in fixed smears, they did not bind to infected erythrocytes in suspension, and so the antigens recognised by these antibodies are probably not externally expressed on infected erythrocytes. Nevertheless, protection was transferred with the anti-merozoite monoclonal antibodies, and it therefore seems that in the case of *P. yoelii*, as with *Plasmodium knowlesi*¹¹, antibody-mediated protection against malaria acts at the level of the free merozoite, inhibiting invasion of host erythrocytes.

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Copper metallothionein, a copper-binding protein from *Neurospora crassa*

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Copper is an essential constituent of many proteins which participate in biologically important reactions¹. In contrast to iron, where different metal storage and transport proteins have been extensively characterised, the existence of copper proteins serving such functions is still a matter of controversy^{2–8}. Studies on the biosynthesis of tyrosinase from *Neurospora crassa* with respect to the copper status of this fungus have shown that this organism accumulates copper with the concomitant synthesis of a small molecular weight copper-binding protein. This protein is now shown to have a striking sequence homology to the zinc- and cadmium-containing metallothioneins from vertebrates⁹. Growth experiments suggest that this molecule fulfills several important physiological functions in this organism such as copper storage, copper detoxification and provision of copper for tyrosinase.

When *N. crassa* is grown in the presence of 0.5 mM of CuSO₄ in the culture medium most of the intracellular copper is found in a high molecular weight fraction during the logarithmic growth period (Fig. 1). However, in the stationary phase about 10% of the copper taken up by the cells accumulates in the form of a low molecular weight protein (Fig. 1). This copper-binding protein was isolated from lyophilised mycelium (obtained as described in Fig. 1 legend) by gel filtration of the crude extract on Sephadex G-50 and subsequent chromatography on DEAE-cellulose. The pure protein is composed of only seven different amino acids with a strikingly high content of cysteine (28%), serine (28%) and glycine (24%). The amino acid composition is: Asx, 2.9; Ser, 6.9; Gly, 6.0; Ala, 1.0; Cys, 6.9 (determined as carboxymethylcysteine); Lys, 1.0. The protein binds a total of 6 g atoms of copper per mw of 2,200. The copper-free apoprotein can be obtained by exposure of the holoprotein to low pH (<pH 1.0) and subsequent metal removal by gel filtration. The homogeneity of the molecule was further corroborated by the

Table 2 IIF titres of sera used in passive transfer experiments

Serum source	Anti- <i>P. yoelii</i> IIF titre
BALB/c mice carrying hybridoma WIC 25.1	1:30,000
BALB/c mice carrying hybridoma WIC 25.23	1:32,000
BALB/c mice carrying hybridoma WIC 25.37	1:128,000
BALB/c mice carrying hybridoma WIC 25.54	1:30,000
BALB/c mice carrying hybridoma WIC 25.77	1:128,000
Immune BALB/c mice*	1:70,000
Normal BALB/c mice	<1:20

Anti-*P. yoelii* IIF titre is expressed as the highest dilution giving positive fluorescence.

* Donors had recovered from mild *P. yoelii* 17X infection, and serum was taken 7 days after challenge with virulent *P. yoelii* (YM strain).

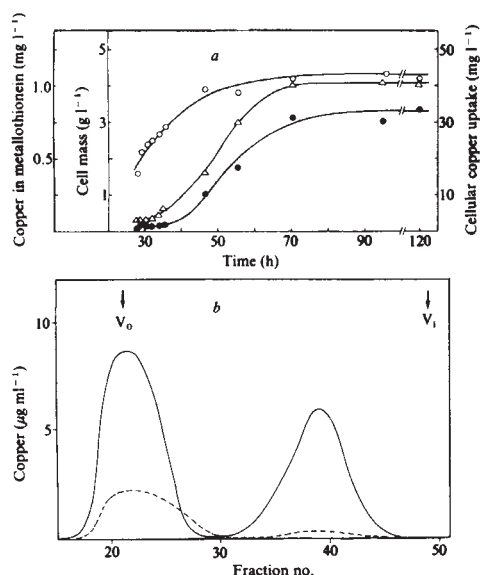


Fig. 1 *a*, Growth curve of *N. crassa* in the presence of 0.5 mM CuSO_4 in the culture medium. The amount of copper metallothionein ($\bullet$), the uptake of copper from the medium (Δ) and the increase of cell mass as dry weight ($\circ$) are shown as a function of time. Cultures of 400 ml half-strength Vogel medium N (ref. 17) supplemented with 0.5 mM CuSO_4 were grown in 1-l Erlenmeyer flasks at 25 °C by shaking at 105 oscillations min^{-1} on a reciprocal shaker. Cells were collected by filtration through cheesecloth, washed with distilled water and the lyophilised mycelium extracted with 50 mM phosphate, pH 7.2. The concentration of copper metallothionein was determined by gel filtration of crude extracts on Sephadex G-50 in 10 mM Tris-HCl, pH 8.0, and subsequent measurement of the metal content of the fraction eluting at an apparent mw of about 3,000. Copper analyses were carried out by atomic absorption spectroscopy. All parameters are expressed per l of culture medium. *b*, Sephadex G-50 elution profiles of crude extracts from 35-h (---) and 70-h (—) old mycelium. Experimental details as in *a*.

What is the state of the copper bound in *Neurospora* metallothionein? Absorption and electron paramagnetic resonance spectroscopy of the freshly isolated protein suggest the copper to be present in the cuprous form. The lack of free cysteine residues and disulphide bridges as well as the broad featureless absorption around 250 nm are indicative of a copper(I)-thiolate binding mode. On the basis of these findings it is proposed that the model for *Neurospora* copper metallothionein is as shown in Fig. 3.

According to this model the net charge of the molecule is expected to be -1 at neutral pH, which is in line with the elution behaviour of the protein on DEAE-cellulose at pH 8.0. The structure is also supported on the grounds of model studies of Cu(I) complexes with sulphur-containing ligands¹⁵, as is the stoichiometry of ligand to metal of close to one.

What is the biological function of *Neurospora* copper metallothionein? Because copper is considered to be a rather toxic element for most organisms¹⁶ the protein could be involved in the detoxification of this metal as was discussed for metallothionein with respect to cadmium⁹. However, the large metal-binding capacity of *Neurospora* copper metallothionein also makes it a good candidate for an intracellular storage protein. This is supported by the results shown in Fig. 4*a*. The amount of copper metallothionein depends on the copper concentration up to approximately 100 μM and then levels off to reach a constant value up to 1 mM Cu in the culture medium. Above 2 mM the growth sharply declines, with a concomitant increase in free ionic copper intracellularly and a decrease in copper metallothionein.

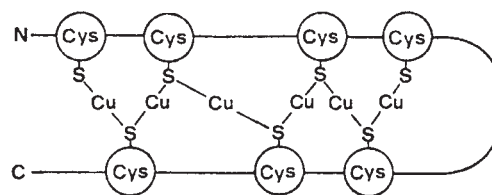


Fig. 3 Proposed model for *Neurospora* copper metallothionein.

These results clearly point to different functions of metallothionein in the copper metabolism depending on the amount of copper present in the culture medium. The complete uptake of the metal ion at low, presumably physiological concentrations of copper and the appearance of relatively large amounts of the metal ion intracellularly in the form of metallothionein (>40% of intracellular copper) is fully consistent with a storage function. On the other hand, the pronounced tolerance towards copper within a rather broad concentration range characterised by a constant copper metallothionein content suggests a detoxification function. At very high concentrations of copper (>3 mM) in the culture medium the toxic effects predominate; this is demonstrated by a sharp increase in intracellular free ionic copper and by a marked decrease in copper metallothionein.

As *Neurospora* copper metallothionein has an exceptionally high cysteine content the relationship between the available sulphur in the culture medium and the amount of copper metallothionein was also investigated (Fig. 4*b*). The data not only demonstrate a correlation between cellular copper uptake and appearance of metallothionein as pointed out above, but also show dependence of the two parameters on the sulphate

determination of its primary structure using automated Edman degradation of the pyridine-ethylated apoprotein¹⁰. Partial sequence analyses of the tryptic peptides derived from the S-aminoethylated apoprotein¹¹ and amino acid analyses of tryptic peptides were in agreement with the results from the automated Edman degradations. The amino acid sequence is strikingly similar to those of the amino-terminal region of the Cd/Zn metallothioneins from man, horse and mouse¹²⁻¹⁴ (Fig. 2). The cysteinyl residues of the *Neurospora* copper-binding protein occupy exactly the same positions as the first seven cysteinyl residues in the amino-terminal sequence region of the Zn/Cd metallothioneins. In addition, serine residue 12 is invariant in all four proteins. Note the complete lack of aromatic amino acids in the copper-binding protein from *N. crassa* as well as in all the Cd/Zn metallothioneins sequenced so far⁹. Because of these common structural features we categorise the *Neurospora* copper-binding protein as a member of the superfamily of metallothioneins⁹. Further, it is suggested that the gene of the *Neurospora* copper-binding protein is related to the primordial gene of the vertebrate metallothioneins which might have evolved to the well characterised present day forms through gene duplication.

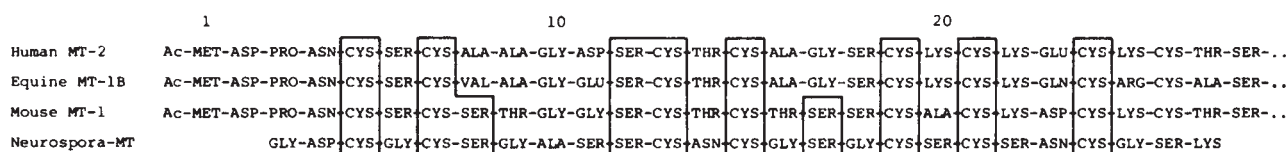


Fig. 2 Comparison of sequences of *Neurospora* copper metallothionein and Zn/Cd metallothioneins from human¹², equine¹³ and mouse¹⁴ livers. Only the amino-terminal portions of the Zn/Cd metallothioneins displaying sequence homology to the *Neurospora* copper metallothionein are shown. Identical residues are indicated by boxes.

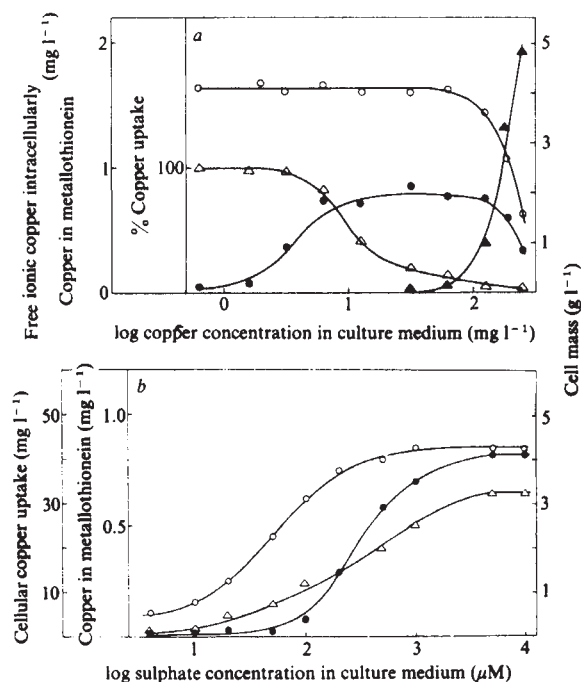


Fig. 4 *a*, The effect of copper added to the medium on the growth of *N. crassa* (○), the amount of metallothionein (●), the percentage of copper taken up from the culture medium (△) and the amount of free ionic copper (eluting at V_i on Sephadex G-50) (▲). Copper was added as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Cells were collected after 72 h and processed as described in Fig. 1 legend. The total amount of copper taken up by the cells was determined by atomic absorption spectroscopy on samples wet-ashed at 120°C for 2 h with an acid mixture made from a 2:1 ratio of concentrated nitric acid to 70% perchloric acid. *b*, The effect of sulphate added to the medium on the growth of *N. crassa* (○), the amount of copper metallothionein synthesised (●), and the copper uptake from the culture medium (△). Copper was added as $\text{Cu}(\text{NO}_3)_2$ at a concentration of 0.5 mM. Sulphate was added as $(\text{NH}_4)_2\text{SO}_4$. Cells were collected after 72 h and processed as described in Fig. 1a legend.

levels in the culture medium. Further, the toxic effects of the copper as manifested by the decreased growth of the fungus are clearly related to the sulphate concentration. These results once more strongly suggest a function of copper metallothionein in copper detoxification.

Finally, copper metallothionein might serve the function of a metal donor to the active sites of copper-containing enzymes. This conjecture is supported by *in vitro* reconstitution studies with *Neurospora* apotyrosinase and copper metallothionein. The complete reconstitution of apotyrosinase (20 μM in 0.1 M sodium phosphate, pH 7.5) with a twofold excess of Cu^{2+} ions at 0°C is a rather slow process (5–10 h); surprisingly, the treatment of apotyrosinase with copper metallothionein in the same conditions is faster by almost an order of magnitude. As the concentrations of intracellular free ionic copper are extremely small in physiological conditions, it is tempting to ascribe to *Neurospora* copper metallothionein an *in vivo* metal donor function.

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Storage enhances chromosome damage after exposure of human leukocytes to mitomycin C

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Two of the distinctive differences between the mutagenic response of *Drosophila* sperm exposed to X rays and to alkylating agents, are: (1) an increase in the incidence of chromosomal structural changes with increasing storage time (days, or weeks) prior to fertilisation following treatments with chemical mutagens, but not X rays, and (2) the frequent occurrence of F_1 individuals mosaic for either mutation or chromosome structural change after chemical treatment, but not after X-ray radiation¹. Similar time-dependent increases in mutations and chromosome aberrations have also been reported for fungal spores² and plant seed root meristem cells³ exposed to alkylating agents, but no enhancing effect of storage could be demonstrated in confluent human fibroblasts exposed to an alkylating agent *in vitro*⁴. We show here that a storage effect can be demonstrated for sister chromatid exchange (SCE) and, to a much greater extent, for chromosome structural changes in human lymphocytes exposed to mitomycin C and stored for various periods before stimulation with phytohaemagglutinin (PHA). Moreover, mitomycin C-treated G_0 cells, which normally develop only chromatid-type aberrations, also yield chromosome-type changes after storage. This result implies a time-dependent alteration in the induced lesions following storage, and predicts a decreasing incidence of mosaicism with increasing storage time—a result that has indeed previously been reported for stored *Drosophila* sperm⁵.

The small lymphocytes in human peripheral blood are in a G_0 state and respond to PHA stimulation *in vitro* with the fast responding cells beginning DNA synthesis 24 h after stimulation and reaching mitosis at around 36 h (refs 6, 7). Following stimulation, cells may proceed through several mitotic cycles, with cycle times of around 16 h, over a period of a week or more in culture (unpublished data). Slow responding cells undergo a delayed transformation to a blast state such that they appear at their first mitosis many days after initial exposure to PHA. Fast responding cells are mainly T cells and slow responders contain a higher proportion of B cells⁸. To study the response of fast and slow developing cells to the induction of SCE and aberrations by mitomycin C, and to examine the effects of storage on induced damage, two types of experiment were performed.

For experiment 1, duplicate sets of blood cultures were set up in 10 ml RPMI 1640 medium (without serum and PHA) using 0.8 ml of heparinised whole blood from three healthy donors. One set was used as a control and the other treated with 6×10^{-7} M mitomycin C (Kyowa) for 1 h at 37°C . The cells were then washed in medium and resuspended in fresh medium with serum, but without PHA, and incubated at 37°C . Lymphocytes were stimulated to divide at 24 h intervals (from 0 to 9 days after treatment) by transferring to complete medium containing 20% fetal calf serum, 1% PHA (Wellcome), 1% glutamine, 100 units

penicillin, 100 µg streptomycin and 25 µM bromodeoxyuridine (BUdR). Stimulated cultures were kept in the dark at 37 °C and cells were collected at 72 h. Air-dried preparations were stained using the fluorescence plus Giemsa technique for differential chromatid staining⁹. With this protocol cells were kept for increasing periods, up to 9 days, in G₀ after mitomycin C treatment and samples of first and second division cells were available for up to 12 days after initial exposure to mitomycin C. Twenty second-division metaphases were analysed on each coded slide to determine SCE frequencies (Fig. 1). For chromosomal aberration incidence, first division metaphase cells which did not show differential staining were analysed from samples stimulated with PHA after 1, 5 and 8 days in culture and then fixed 72 h later (Table 1).

In the second experiment PHA and BUdR was added at the initiation of culture, the medium replaced with fresh complete medium every 72 h, and cells collected at daily intervals up to 7 days. The experiment was performed in two parts, and the SCE frequencies determined on coded slides are summarised in Fig. 2. The numbers of first division cells in late cultures were too small to give useful data on aberration frequencies.

The incidence of SCE in control cultures in experiment 1 showed no significant difference between donors or between culture times over a 9-day period. Mitomycin C exposure gave a typical eightfold increase in SCE levels over controls in cells stimulated over the period 1–4 days, but this was followed by a further significant increase (up to 100%) to a peak level at 6–8 days and a fall at 9 days. In cultures stimulated at time zero and sampled serially thereafter (experiment 2), there were no inter-donor differences and control samples showed a small increase in SCE with time in culture. Following mitomycin C treatment, there was again a rise in SCE frequency with time after exposure.

A trivial explanation of the increase in SCE with time after mitomycin C treatment would be that cells coming into their second mitosis around their sixth day in culture, whether stimulated at time zero and therefore 'slow' cells (experiment 2), or at day 4 and 'fast' cells (experiment 1), are more sensitive than other cells. This seems highly unlikely, since the samples scored

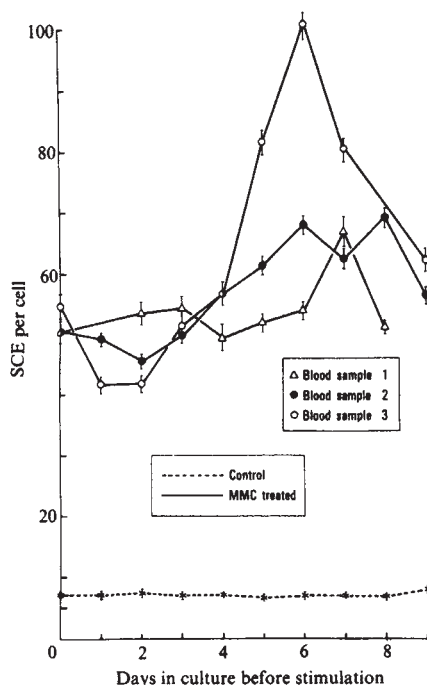


Fig. 1 Incidence of SCE (20 second-division cells per point) in lymphocytes from three donors exposed to 6×10^{-7} M mitomycin C for 1 h before culture and then stimulated with PHA at different times after treatment. Broken line, control; solid lines, mitomycin C-treated. Δ , Blood sample 1; $\bullet$, blood sample 2; $\circ$, blood sample 3.

Table 1 Chromosomal aberrations at the first division in cells treated with mitomycin C and then stimulated with PHA after various intervals in culture

Day of addition of PHA:	Controls			Exposure to mitomycin C*		
	1	5	8	1	5	8
Total metaphases analysed	150	150	150	150	140	100
Abnormal cells	4	5	4	16	19	40
Chromatid gaps	1	1	2	1	1	13
Chromatid breaks				4	3	7
Chromatid interchanges				1	5	9
Total chromatid breaks + exchanges (per cell)	0	0	0	0.033	0.057	0.160
Chromosome gaps† (isolocus)				2	2	6
Chromosome fragments†				3	7	11
Dicentric					2	2
Rings					1	1
Symmetrical intra- and interchanges						4
Total chromosome breaks + exchanges (per cell)	0	0	0	0.020	0.071	0.180

* Exposure (1 h) to 6×10^{-7} M mitomycin C at zero time.

† The categories of chromosome gaps and fragments may include some chromatid-type isolocus events.

at these times represent different cell populations. To confirm this point, in a third experiment we exposed cells either to PHA plus mitomycin C at time zero and sampled at 3 and 4 days, or to PHA plus mitomycin C at day 4 and sampled on days 7 and 8. The mean frequencies of SCE, and of chromatid aberrations, at days 3 and 4 did not differ from those found at days 7 and 8 (Table 2), so that the latter cells were not more sensitive than the former. There is, however, evidence of differences in response between B and T lymphocytes to the induction of SCE by BUdR, with the former showing a lower response¹⁰. Since B cells may predominate in very late cultures, this may account for a decline in SCE frequency at this time, but we have no evidence of a differential sensitivity of B and T cells to damage by alkylating agents. We conclude, therefore, that the mitomycin C-induced increase in SCE with time after treatment is a time-dependent phenomenon that is not a consequence of differential sensitivity, but is a true storage effect.

The data on mitomycin C-induced aberration frequencies in first division cells, that is, non-harlequin-stained metaphases, are summarised in Table 1. Two significant findings are evident: first, there is a marked increase in aberrations with time after mitomycin C treatment, the incidence following PHA addition on day 8 being seven times greater than on day 1; second, this increase is contributed to almost equally by chromatid- and chromosome-type aberrations. It is well established that proliferating somatic cells exposed to alkylating agents contain only chromatid-type aberrations at their first post-treatment mitosis. These aberrations, in which the unit of exchange or breakage is the single chromatid, are produced following exposure of cells at any stage in the cell cycle, and are a consequence of a mis-replication at sites of damage during the replication phase^{11,12}. In contrast, X-ray exposure of unreplicated chromatids in the G₀ stage yields chromosome-type aberrations, where breakage and exchange occurs prior to replication and the aberrant structure is itself replicated at the S phase¹³. Lymphocyte nuclei exposed to mitomycin C are in a G₀ state at the time of treatment and in all short term cultures the only aberrations seen at mitotic first

Table 2 Chromatid aberrations and SCE frequencies in cells exposed to 6×10^{-7} M mitomycin C for 1 h at zero hours or after 4 days in culture

Time of mitomycin C treatment		0 h		96 h	
Sampling time post-treatment		72 h	96 h	72 h	96 h
Chromatid aberrations per first division cell*	Controls	0	0.01	0	0
	Mitomycin C-treated	0.05	0.08	0.05	0.11
SCE frequencies in second division cells†	Controls	7.35	9.0	7.15	9.0
	Mitomycin C-treated	53.1	57.8	51.9	55.5

* 100 first-division cells analysed at each point.

† 40 second-division cells analysed at each point.

division in the first days following exposure are of the chromatid type. The presence of chromosome-type changes in first division cells when stored for periods of 5 to 8 days was therefore entirely unexpected.

Our demonstration of a large increase in chromosome aberrations and of a change in aberration type with increasing storage time following exposure to an alkylating agent, parallels earlier findings on *Drosophila* sperm. An aberration affecting a single chromatid in a fertilisation nucleus will segregate at mitosis to one of the daughter cells and may result in an individual mosaic for the abnormality, whereas a chromosome-type aberration, if viable, would be present in all descendent cells giving a whole-body mutant. Our result suggest that increased storage should result in a decreased proportion of mosaic mutants recovered and such a result has been reported for *Drosophila* sperm treated with triethylene melamine⁵.

We had previously shown that chromatid-type aberration frequencies in second division cells after exposure to alkylating agents are higher than in first division cells^{11,12}. This is consistent with the present findings and implies changes in the damaged DNA with time post-exposure. The interaction of mitomycin C with DNA is complex, although it is known that the semiquinone radical formed during its reduction in the cell binds very rapidly to DNA, particularly to the guanine moiety, and that the predominant site of alkylation is probably at the O^6 of

guanine¹⁴. Early changes in chromatid aberration frequencies may partly reflect single strand breakage due to chain instability following hydrolytic loss of alkylguanine, breakage arising either due to increased lability at the depurinated sites or to the action of an endonuclease II-type enzyme¹⁵. Mis-replication at sites of single strand damage may result in chromatid-type aberrations at replication, but chromosome-type aberrations require the presence of double-stranded scissions before replication¹⁶. Chain breakage following depurination may be a rare event, but chromosome-type aberrations may result from the coincidence of two single strand breaks that develop as secondary consequences of the alkylation that occurred many days earlier. Whatever the nature of the changes in lesions with time after exposure, these are more effective in increasing aberration frequency and result in only small increases in SCE.

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Partial nucleotide sequence of the 300-nucleotide interspersed repeated human DNA sequences

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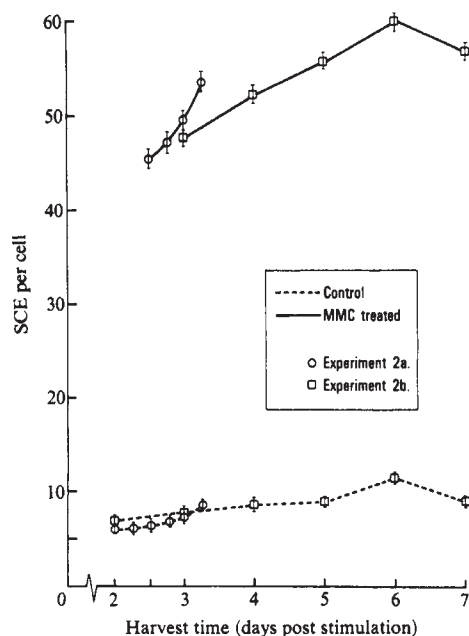


Fig. 2 Incidence of SCE (60 second-division cells per point) in lymphocytes from three donors exposed to 6×10^{-7} M mitomycin C for 1 h and then immediately cultured in the presence of PHA and serum. Broken lines, control; solid lines, mitomycin C treated. ○, Experiment 2a; □, experiment 2b.

In most eukaryotic genomes, including human, 300-nucleotide repeated DNA sequences are interspersed with longer (~1,000 nucleotide) single copy sequences¹⁻³. We have recently found that most 300-nucleotide interspersed repeats in human share a common site for cleavage by the restriction enzyme *Alu* and should be regarded as a single family of sequences⁴. We designate this as the *Alu* family of sequences. Similarly, most of the 300-nucleotide inverted repeated sequences, which are also interspersed with single copy DNA, share this same restriction site and belong to the *Alu* family⁴. There are approximately 300,000 members of this family of sequences, which together make up at least 3% of the human genome⁴. It is conceivable that individual members of the *Alu* family repeats share only very limited regions of homology, one of which happens to contain the restriction site for *Alu* and others which share the additional restriction sites reported here. In this case, members of the *Alu* family could be essentially different DNA sequences. DNA renaturation studies support the alternative view that members of the *Alu* family share extensive homology over the entire sequence length⁴. According to this view, individual members of the family could exhibit some divergence from the



As one control, we compare the fidelity of the sequence and its complement (Fig. 2). Complementary sequences were obtained as shown in Fig. 1a. In one portion of the proposed average sequence (positions -92 to -30) there are 11 differences in 62 assignments, which corresponds to an 18% sequence mismatching. In the other portion of the sequence (positions +12 to +69) there is 5% mismatching (3 differences in 56 assignments) between the sequence and its complement (Fig. 2). These values compare favourably to estimates from thermal stability studies which indicate an average of 12% mismatching in renatured interspersed repeated human DNA sequences⁵.

As a second control on the accuracy of this sequence we have also determined the base sequence of some cloned members of the *Alu* family. Clones were constructed by ligating synthetic *Bam*H1 linkers on the 300-nucleotide repeated sequences described above using a modification of a procedure used to clone sea urchin repeats⁷. These were propagated by insertion into the *Bam*H1 site of the episome pBR322 and grown under P3, EK1 containment.

One clone, clone 8, was base sequenced in its entirety; another, clone 2, has been partially sequenced. The sequencing techniques used for this determination are summarised in Fig. 2, and we regard the assignments as being unambiguous. The sequences of these two clones are closely related to each other and to the average base sequence described above (Fig. 2). With the exception of a deletion in clone 8 at position 33, the overall sequence mismatching between clones 2 and 8 is 24% (27 differences in 113 shared sites). Twelve of these mismatches are clustered near position +75. A more extensive comparison of additional cloned sequences is required to establish whether

mutations are randomly distributed or clustered at certain preferred sites. It is especially interesting to compare the relative sequence fidelity of the two clones and the *Alu* family in the region shared by all three sequences, positions +3 to +69. There is 18% mismatching (11 differences in 61 sites) between clones 2 and 8 in this region. There is 22% mismatching (14.5 differences in 67 sites) between the *Alu* family and clone 8 and 21% mismatching (13 differences in 62 sites) between the *Alu* family and clone 2 in this shared region. The agreement between these estimates suggests that the average sequence proposed for the *Alu* family resembles the actual sequence as closely as any individual cloned member of the family. As previously noted, we expect 12% mismatching of renatured interspersed repeated human DNA⁵. Our representation of the *Alu* family therefore approximates the fidelity with which the sequence has been evolutionarily conserved.

Jelinek and co-workers have found that the repetitive double strand regions in HeLa cell heterogeneous nuclear RNA (hnRNA) have a very simple fingerprint⁸⁻¹⁰ dominated by six prominent oligonucleotides. This implies that repetitive hnRNA may contain one or at most a few distinct families of sequences⁸⁻¹⁰. In support of this conclusion, we find that five of the six oligonucleotides (oligonucleotides 1, 2, 3, 5, 6 reported by Jelinek *et al.*⁸) are present or approximately represented in the *Alu* family of sequences (Fig. 2). The remaining oligonucleotide 4 was later found to be a mixture of sequences and was not further characterised⁹. We find two sites in the *Alu* family which resemble oligonucleotide 4 and may be the composite source of that sequence (Fig. 2). The *Alu* family of repeated DNA sequences is sufficient to account for part if not all of the repetitive hnRNA sequences described by Jelinek and his colleagues⁸⁻¹⁰. As further evidence for this conclusion, Jelinek observed the same six prominent oligonucleotides in fingerprints of *in vitro* transcripts of inverted repeated DNA sequences⁹. As noted above, the 300-nucleotide inverted repeated sequences are members of the *Alu* family and should, as Jelinek observed, also contain these oligonucleotides.

We have restricted our description of this family of sequences to the human genome. However, work in progress in our laboratory indicates that a closely related family of sequences is found in all primate genomes (galago, monkey and human). Furthermore, Jelinek's observation of a single prominent oligonucleotide in the repetitive double-strand regions of hamster hnRNA is sufficient evidence to suggest that, in analogy to human, the hamster genome is also dominated by a single family of interspersed repetitive DNA sequences¹¹.

The biological function of this family of sequences is unknown. We and our colleagues have recently noted sequence similarities between a selected portion of the *Alu* family and several other RNA or DNA sequences, which are known or suspected to be involved in DNA replication, transcription control, and mRNA processing¹². Together these observations reinforce our belief that a family of DNA sequences which includes 300,000 highly conserved members interspersed throughout much of the mammalian genome, must have an important function.

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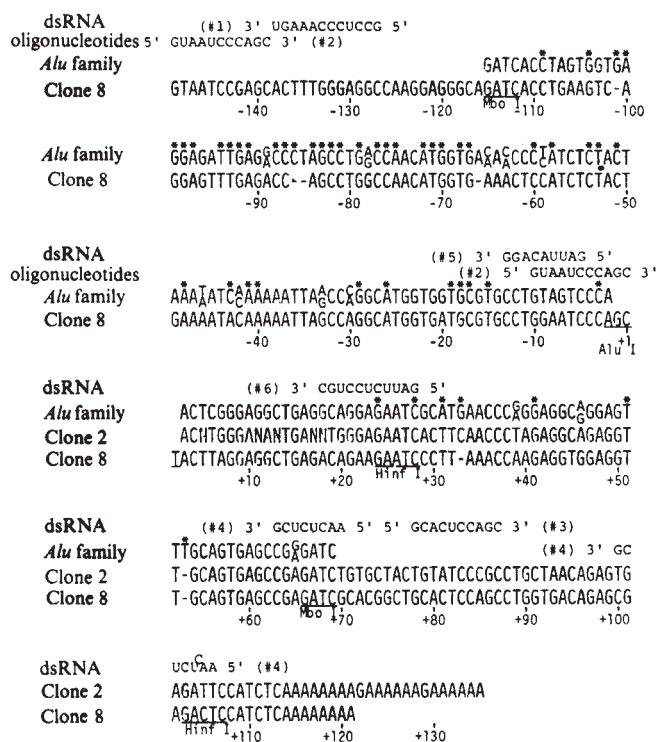


Fig. 2 The sequences of the *Alu* family and clones 2 and 8 are shown in large type. Since the ends of the sequence are not precisely defined, the sequence is numbered in both directions from the *Alu*I site. The method for determining the average sequence of the *Alu* family is described in the text and Fig. 1a. Asterisks designate sites at which the readings of either the sequence or its complement were ambiguous (see Fig. 1b). Positions at which the sequence and its complement did not agree are indicated by the presence of two bases. In the base pair fidelity calculations presented in the text these positions are assigned a value of 0.5 (that is, half mismatched). Clone 8 was cleaved at the artificial *Bam*H1 linkers (see text), fill-in labelled at the 3' ends using α -³²P and reverse transcriptase and strand separated on a polyacrylamide gel. It was then sequenced by the Maxam-Gilbert technique⁶. Clone 2 was cleaved at the synthetic *Bam*H1 sites, labelled at the 5' end with γ -³²P and polynucleotide kinase and cleaved with *Alu*I. The sequence between the right *Bam*H1 site and the *Alu*I site was determined by the Maxam-Gilbert method. All the DNA sequences read from 5' to 3'. A deletion in one of the sequences compared to another is indicated as (-). The dsRNA oligonucleotides are shown in smaller type. The sequences of these oligonucleotides (1 to 6) are from the results of Jelinek and coworkers⁸⁻¹⁰. The polarity of the RNA strands is indicated in the figure.

MATTERS ARISING

Bragg intensities near structural phase transitions

IN a letter to *Nature* Åsbrink and Hong reported an increase of X-ray reflection intensity and profile widths at the low-to-high- V_2O_5 phase transition state¹. The oldest reference I know of to the increase of X-ray intensity near structural phase transitions is that of De Quervain² in 1944. The effect was observed in a ferroelectric material (KDP) of which large rather perfect crystals can easily be grown. As these crystals show severe extinction, any disturbance caused by a phase transition is liable to increase the reflectivity. Since then, this effect has been observed rather often near first-order phase transitions, and also with neutron or γ -ray scattering³. It has been used as a very accurate indication for the occurrence of the transition^{4,5} in a pressure cell. (We commonly use a series of crystals with various transition temperatures for accurate cryostat calibration.)

As this overshooting concerns integrated intensities, the width of the reflection profiles also increases. Various authors have used the information which lies in the variation near the transition of the profile shapes to characterise the state of the crystal near T_c . For example, Zeyen *et al.*^{6,7} have studied the reflection profiles of DKDP crystals during its ferroelectric first-order phase transition using high precision cryogenics⁸ and high resolution neutron diffraction techniques⁹. They investigated the spatial distribution of the phase mixing at T_c where both the paraelectric and the ferroelectric phases coexist in the crystal in a particular arrangement which actually minimises elastic and electrostatic (polarised domains) energies and depends on the structural change at T_c . Following their model the DKDP crystal forms a multi-layer of alternating paraelectric and ferroelectric sheets. It explains directly the overshooting intensity near T_c . The existence of these layers has in the meantime been confirmed directly by optical observation in a special cryostat¹⁰. Other systems giving different spatial arrangements have also been studied.

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ÅSBRINK AND HONG REPLY—We thank Zeyen for information on early discussions about intensity overshoot in connection with studies on KH_2PO_4 and KD_2PO_4 .

The possibility of phase mixing during the transition occurred to us; however, we had to discard it as several reflections did not even exhibit the profile widening at $t_T = 154.7^\circ C$ required by the difference in unit cell dimensions between the two V_2O_5 phases. (We made the comparative profile width measurements at 153 and $156^\circ C$ for low- and high- V_2O_5 , respectively, and assumed that the entire change of unit cell dimensions between those temperatures took place at t_T .)

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Magnetostatigraphy, biostratigraphy and geochronology of Cretaceous-Tertiary boundary sediments, Red Deer Valley

LERBEKMO *ET AL.*¹ have presented some valuable radiometric data from an important continental sedimentary sequence which includes the Cretaceous-Tertiary boundary in Alberta. However, the palaeomagnetic data which they presented in their Fig. 2 show considerable scatter and do not provide a well defined polarity zonation.

Lerbekmo *et al.* correlate their magnetic polarity zonation from the Red Deer Valley with the magnetic polarity time scale. They note that the Cretaceous-Tertiary boundary, as recognised by marine biozonations, occurs near the base of anomaly 29. Lerbekmo *et al.* then concluded that the long normal polarity zone overlying the Cretaceous-Tertiary boundary in the Red Deer Valley, as recognised by dinosaur extinction and palynofloral zones, must correlate with anomaly 29. We consider this correlation to be circular reasoning based on the incorrect presumption that the Cretaceous-Tertiary boundary in the Red Deer Valley is synchronous with the Cretaceous-Tertiary boundary as determined by marine biozonations. This normal polarity zone in the Red Deer Valley could correlate with anomaly 28 or even anomaly 27. The magnetic polarity zonation in the Red Deer Valley does not show a convincing correlation to the magnetic polarity time scale. Thus, the conclusions reached by Lerbekmo *et al.*

based on their correlation are rather speculative.

In addition, Lerbekmo *et al.* proposed an alternative to our^{2,3} correlation between the San Juan Basin magnetic polarity zonation and the magnetic polarity time scale. They suggested that the normal polarity zone which we correlated with anomaly 29 should be correlated with a normal polarity interval between anomalies 29 and 30. However, there is no evidence for such a normal polarity interval in either of the two magnetostratigraphic sections at Gubbio, Italy^{4,5} nor in the magnetostratigraphic section at Moria, Italy⁶ nor in the marine magnetic anomaly record⁷. The palaeomagnetic data from the San Juan Basin provide a well defined magnetic polarity zonation which shows a strong correlation with the magnetic polarity time scale^{2,3}. We do not agree with Lerbekmo *et al.*'s reinterpretation of our data.

Lastly, we do not agree that there are "palaeontological discrepancies between the New Mexico, Alberta, and Gubbio, Italy sections with respect to the Cretaceous-Tertiary boundary". As we pointed out², the Cretaceous-Tertiary boundary is defined by extinction of marine invertebrates but is recognised in terrestrial sedimentary sequences by the last occurrence of dinosaur fossils. These two biological events need not have occurred synchronously and marine/non-marine intertonguing relationships do not provide enough precision to test for global synchronicity of this geological-time boundary. However, magnetostratigraphy does provide a possible technique for determining the temporal relationship of dinosaur extinction and marine invertebrate extinctions marking the Cretaceous-Tertiary boundary. Thus our magnetostratigraphic data which indicate a lack of synchronicity between dinosaur extinction in the San Juan Basin and the Cretaceous-Tertiary boundary at Gubbio, Italy do not constitute a palaeontological discrepancy as implied by Lerbekmo *et al.*

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LERBEKMO ET AL. REPLY—Butler and Lindsay have focused attention on some of the problems in magnetostratigraphic correlation. They point out that our palaeomagnetic data show considerable scatter and do not provide a well defined polarity zonation. This reiterates the statement in our original article. Nevertheless, there is a stratigraphic zonation present—the data are not randomly scattered. We believe the key to interpretation of this type of data is the recognition of the importance of viscous and/or chemical overprinting; nature has not provided ideal material and we have attempted a rational interpretation following the reasoning of Hillhouse *et al.*¹. A closely comparable study involving the same problems, and following essentially the same scheme has been reported by Brown *et al.*². We are convinced that a real pattern of normal and reversed polarity emerges from our data, although some overprinting may still be present.

Butler and Lindsay suggest that we have used circular reasoning in our anomaly matching, which we have not. This can probably be attributed to the limited palaeontological background information contained in our original article. In fact, we believe that our correlation of the Red Deer Valley section with the Gubbio section in Italy is unambiguous for the following reasons.

The palynomorph change which takes place just above the Nevis coal seam in Alberta³ and at the Hell Creek (Lance)—Fort Union contact in Montana and Wyoming³, also takes place at the Hell Creek—Ludlow (Lignite Beds) contact in North Dakota in the type area of the Cannonball Formation⁴. The Ludlow there is 5–20 m thick and is overlain by the marine Cannonball which reaches a thickness of 125 m in the subsurface at Garrison Dam⁵. Foraminifera recovered from the Garrison Dam core place the entire formation in the *Globigerina edita* zone, which is the lowest Palaeocene foraminiferal zone and is equated⁵ to the *Globorotalia pseudobulloides* zone plus the thin *Globigerina eugubina* zone (which occur in the Gubbio section). The Cannonball Formation spans the *Globorotalia pseudobulloides* zone and is separated from the Cretaceous–Tertiary boundary below, as defined by palynomorphs, by about 20 m of Lignite Beds (=Ludlow)^{4,5}. The *Globorotalia pseudobulloides* zone at Gubbio encompasses part of anomaly 28, all of anomaly 29 and part of the reversed zone between anomalies 29 and 30 (ref. 6). Therefore, if the palynomorph change equated with the Cretaceous–Tertiary boundary in Alberta, Montana, North Dakota and Wyoming³ is essentially synchronous throughout this area, as believed⁴, this palynomorph boundary and the highest occurrences of dinosaurs are in the same reversed zone in which the foraminiferal Cretaceous–Tertiary boundary takes

place in Italy (that is between anomalies 29 and 30).

The anomaly matching by Butler *et al.*⁷ and Lindsay *et al.*⁸ is not infallible; whether or not the results summarised in Fig. 2 of ref. 8 constitute a “strong correlation with the magnetic polarity time scale” is debatable. The problem of ‘extra’ polarity intervals arises. In their comment above Butler and Lindsay question the validity of an additional normal interval between 29 and 30. However, in Fig. 2 of ref. 8 two ‘extra’ polarity intervals are shown which do not appear in the seafloor polarity record⁹. We expect that detailed palaeomagnetic studies of this type will reveal short magnetic zones not now recognised in the seafloor anomaly pattern, but much further work will be required to separate these from fictitious overprinted intervals.

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British Tertiary Igneous Province probably not associated with East Greenland lavas

In their recent article Carter *et al.*¹ discuss the source regions of continental basalts extruded round the North Atlantic in the early Tertiary. They made the common assumption that the British Tertiary Igneous Province (BTIP) was formed as a result of the rifting which led to the separation of Greenland from Europe. Their reason for this assumption was presumably that the formation of the BTIP was close in space and time to the

rifting. However, this similarity may be more apparent than real and be misleading by concealing significant differences, summarised below.

Carter *et al.* give the duration of activity in the BTIP as 66–50 Myr ago. Although such a spread of dates—and greater—can be found in the literature, note that the province has proved particularly difficult to date accurately, and many published dates are inconsistent with the stratigraphy, for example K–Ar dates for Faeroes² and Mull dykes³. If only the most reliable dates are used, which means chiefly ⁴⁰Ar/³⁹Ar plateau and Rb/Sr isochron ages, most activity is found to have occurred about 60 Myr ago (Table 1). (All dates quoted here have been adjusted to the decay constants and so on of Steiger and Jäger⁴.) Only Lundy is clearly younger.

The time of opening of the North-east Atlantic is not yet agreed, but is usually assumed to have just preceded the formation of magnetic anomaly 24. The age of this anomaly is deduced by calibrating the anomaly pattern of Heirtzler *et al.*⁵ at various points; because the spreading rate was probably not uniform this leads to a range of estimates for the age of anomaly 24. However, polarity time scales which have been calibrated in the Palaeocene^{6–8} assign it ages of 50.3, 53.4 and 54.4 Myr, respectively. Although the Greenland basalts must be somewhat older than this, it seems unlikely that they are contemporaneous with the BTIP, with the possible exception of Lundy.

Another difference is that activity on the British side of the North-east Atlantic-to-be occurred in discrete areas. Most of these areas lie roughly on a line joining the Faeroes to Lundy (St Kilda and Rockall lie well to the west) which diverges at an angle of ~55° from the margin of the Atlantic, according to the reconstruction of Bullard *et al.*⁹. Thus Arran, for example, is a perpendicular distance of ~300 km from the margin, while Lundy (the part of the BTIP most likely to be contemporaneous with the rifting) is over 500 km distant. In contrast, the Greenland activity lies within 150 km of the margin.

The East Greenland coast has a flexure and associated dyke swarm. In contrast, the BTIP has no flexure and the regional dyke swarms of the separate areas, although roughly parallel to each other, are *en echelon*; these swarms are not what

Table 1 ⁴⁰Ar/³⁹Ar plateau and Rb/Sr isochron dating

Area and rock	Method	Age (Myr)	Ref.
Arran: Northern Granite	⁴⁰ Ar/ ³⁹ Ar plateau	60.4 ± 0.6	10
Mournes: granite	⁴⁰ Ar/ ³⁹ Ar plateau	59.7 ± 1.6	10
Mull: dyke cutting Loch Bà Felsite	⁴⁰ Ar/ ³⁹ Ar plateau	60.3 ± 3	10
Mull: centre 3 granite	Rb/Sr isochron	58.2 ± 2.5	11
Antrim: basalts	K/Ar isochron	~60	12
Lundy: granite	Rb/Sr isochron	53 ± 2, 55 ± 3	13
Lundy: granite	⁴⁰ Ar/ ³⁹ Ar plateau	53.4 ± 1.4	14

would be expected along, or beside, a simple tensional split but rather, say, what would result from a southward movement of Ireland relative to Great Britain.

Most of the activity of the BTIP probably preceded the opening of the North-east Atlantic and the Greenland activity by several million years; it does not occur along the margins of the rift, and occurred in a stress field that does not seem to be related to that of an opening ocean.

These observations do not affect the conclusion of Carter *et al.*, that there is no evidence for an undepleted mantle source-region for any of the basalts they discussed. However, their assumption that the BTIP is simply due to the formation of the North-east Atlantic oversimplifies our understanding of how the BTIP formed. Here it is relevant that they point out that the BTIP may be anomalous as there may have been "overall higher temperatures in the lower crust in North-west Scotland at the time of basalt eruption than elsewhere in the North Atlantic Tertiary Province".

Perhaps it just was not part of this province!

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Cubomedusae belong to the class Cubozoa, not Scyphozoa

IN their report on the neurophysiology of *Carybdea rastonii* Haacke (a cubomedusa), Satterlie and Spencer¹ emphasised that "... properties of the neuromuscular system ... clearly show it to be of the scyphozoan type ...". They also name the Cubomedusae as an order within the class Scyphozoa according to Haeckel's taxonomic scheme². Since the discovery of the first polyp of a cubomedusa³, Werner^{4–7} and Chapman^{7,8} have made extensive studies of the anatomy, behaviour, life history and systematic position of members of this group. The results of these studies led Werner to give

the Cubomedusae the rank of a class (Cubozoa)^{4,5}. This conclusion was supported by Chapman's study of the microanatomy of a cubopolyp⁸.

The principal factor which has led to the new classification is the structure of the polyp. Those cubopolyps which have been studied (*Tripedalia* and *Carybdea*) differ from polyps of the class Scyphozoa in that they lack, among other features, the gastric septa and tetramerous symmetry which are diagnostic of scyphopolyps^{5,6,8–10}. The cubopolyps share radial symmetry, lateral budding, capitate tentacles and other important features with hydrozoan polyps and also possess unique features^{5,6,8}. The neuromuscular system (which Satterlie and Spencer¹ equate with that of the Scyphozoa), like the features mentioned above, clearly illustrates the mixture of unique, scyphozoan-, and hydrozoan-like features in the Cubozoa. For example, it has long been known that cubomedusae possess a marginal nerve ring^{2,9}, like the hydromedusae but unlike the scyphomedusae. A unique feature of the Cubozoa is the presence of an ectodermal-endodermal nerve-ring pair in the polyp⁸. Although Satterlie and Spencer¹ cite the paper by Werner *et al.*⁶ on the neuromuscular system of a cubopolyp, they discuss in their own article only the medusa of *Carybdea* and only scyphozoan-like features of the medusa.

When one is dealing with organisms with a complex life history, such as coelenterates, one must bear in mind all stages of the life cycle if one wishes to draw conclusions about taxonomic position. Satterlie and Spencer's¹ fascinating findings on the neurophysiology of *Carybdea* do not support the retention of the Cubomedusae in the class Scyphozoa but do provide further support for Werner's^{4,5} theory that the Cubozoa are intermediate between the Scyphozoa and Hydrozoa and that the Scyphozoa are the class most similar to the ancestors of the three groups.

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SATTERLIE AND SPENCER REPLY—
Our article was not intended to give

definitive evidence for the taxonomic position of the Cubomedusae. However, judged by the neuromuscular properties of the swimming system of *Carybdea*, the medusae strongly resemble the scyphomedusae. This similarity is so convincing as to make it unnecessary to create a new class, the Cubozoa, to accommodate any unique features. In addition, the structure and function of the swimming system of *Carybdea* is unlike any known hydromedusan system.

The one cubomedusan feature frequently considered hydrozoan-like, the subumbrellar nerve ring, is not structurally or functionally similar to the marginal nerve rings of hydromedusae. In the latter, swimming pacemakers are distributed throughout the inner nerve ring, as an electrically coupled network of 'giant' neurones. In both cubomedusae and scyphomedusae, the swimming pacemakers are restricted to the rhopalial. Transmission of pacemaker output throughout the subumbrellar muscle sheet in both cubomedusae and scyphomedusae is through networks of large bi- and tri-polar neurones, making up subumbrellar nerve nets. Although subumbrellar nerve nets are present in some hydromedusae, the subumbrellar muscle cells show widespread intercellular electrical coupling, and in some cases, make up a conducting musculo-epithelium. Ultrastructural evidence argues against such electrical coupling in cubomedusae. Each swimming contraction of hydromedusae is an all-or-none event, accompanied by an all-or-none action potential in the muscle sheet. In comparison, both cubomedusae and scyphomedusae exhibit graded muscle contractions, and at least in *Carybdea*, graded muscle potentials. As this short comparison points out, not only is there a lack of similarity between the hydromedusan and cubomedusan swimming systems but also a lack of originality on the part of the cubomedusae, as all features are distinctly scyphozoan-like.

As Leonard points out, most of the evidence for proposing a new class comes from work on the structure of cubopolyps. It is difficult to determine whether some of the structural characteristics of cubopolyps, such as the lack of tetramerous symmetry and gastric septa, are primitive features or secondarily derived from a true scyphozoan ancestor. Cubomedusae exhibit structural specialisations in both stages of their life cycle; however, at least in the medusa generation, these specialisations are morphologically and physiologically consistent with the scyphozoan line.

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Enhanced excision of O^6 -alkylguanine in rat liver by pretreatment with acetylaminofluorene

AS recently reported by Buckley *et al.*¹, a diet containing 0.06% 2-acetylaminofluorene (AAF) established in rats a condition in which the liver of the animals had an increased capacity for removing O^6 -methylguanine from DNA produced by a single dose of dimethylnitrosamine (DMN). These observations are open to many interesting interpretations. However, the authors concentrated exclusively on the concept of induction of specific repair enzymes by the presence of damage from AAF adducts. This interpretation does not pay due attention to the biological state of the cell population in the liver at the time of the investigation.

Chronic ingestion of a liver carcinogen such as AAF will stimulate the liver into hyperplasia. Although the authors specifically excluded animals that had nodular livers, it is likely that livers of all animals at the end of this chronic treatment will contain an increased proliferative population^{2,3}. Passage between the non-cycling and proliferative phases of a cell population alters the activity of many enzymes involved in DNA replication and repair. Peripheral lymphocytes stimulated to proliferate by phytohaemagglutinin increase their capacity for repair⁴ and synthesise a specific repair enzyme, uracil glycosylase⁵. In the rat liver, DNA polymerase β , which may participate in excision repair, increases during regenerative hyperplasia following partial hepatectomy⁶. The repair systems do not all respond equally, because the system that removes O^6 -methylguanine increases during liver hyperplasia after chronic treatment with DMN, whereas that for removing N^3 -methyladenine or N^7 -methylguanine does not⁷. Excision repair seems to be particularly responsive to the proliferative state of a cell population^{4,8-10}. One interpretation of the observations of Buckley *et al.*¹ may therefore simply be that they represent the differences between the repair capacity of a quiescent relative to a proliferating cell population. The increased repair of O^6 -methylguanine would not relate specifically to signals from damaged sites inducing the synthesis of new enzymes, but rather to a more generalised consequence of stimulation of proliferation.

Buckley *et al.* imply that exposure to AAF induces the repair of lesions formed by an unrelated carcinogen, DMN. The lesions formed by AAF and the O^6 -methylguanine produced by DMN are not, however, as different as might be expected if one considers the pathways by which they are repaired. Both kinds of lesion can be repaired by the nucleotide excision repair pathway¹¹. This system removes pyrimidine dimers produced by UV light and is regulated by the genes

associated with the human disease xeroderma pigmentosum (XP)¹². The autosomal recessive mutations in XP eliminate the capacity to remove O^6 -methylguanine, O^6 -ethylguanine, pyrimidine dimers, and the lesions produced by *N*-acetoxy-AAF¹³⁻¹⁶. Interestingly, O^6 -methylguanine is not repaired by the nucleotide excision repair system in *Escherichia coli*, although it is in mammalian cells^{15,16}, suggesting that prokaryotic systems may be very poor models to use in the interpretation of observations of O^6 -methylguanine in mammalian cells.

Buckley *et al.* cite evidence for the induction of post-replication repair by *N*-acetoxy-AAF-treated Chinese hamster cells¹⁷, but that observation only demonstrated alterations in semi-conservative DNA replication confined to the first few hours after an acute dose. Those alterations involved the growth of nascent DNA chains, and did not involve any change in the repair of lesions on the parental DNA; also, they have been interpreted elsewhere in a manner that does not invoke the induction of any system¹⁸.

Therefore, we suggest that a change in the excision capacity of liver by pretreatment with AAF more probably reflects the production of a spectrum of enzymes associated with cell proliferation, especially for DNA replication and repair, than the specific induction of unique repair enzymes. Moreover, we should also point out an interesting implication of observations of Buckley *et al.* in relation to the role of O^6 -methylguanine as a critical lesion for carcinogenesis by alkylating agents¹⁹. When rats were given the same feeding regimen of AAF and DMN, the subsequent yield of hepatocellular carcinomas reached nearly 100%, whereas a single dose of DMN given alone produced no liver cancers²⁰. Thus, increased repair of O^6 -methylguanine correlates with increased susceptibility to hepatocarcinogenesis, whereas the opposite correlation prevails in the neonatal rat brain, in which decreased repair of O^6 -methylguanine correlates with elevated carcinogenesis²¹. These contrasting correlations indicate that any attempt to ascribe a unique, definite role to O^6 -alkylguanine in carcinogenesis is premature; the role of this product and its repair are not yet fully understood. At the very least, the balance between DNA repair and DNA replication in each experimental situation needs to be carefully considered.

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BUCKLEY, O'CONNOR AND CRAIG
REPLY—Although more detailed information will be available when a fuller report is published on the basis of our recent study¹, two comments should be made on the matters raised by Cleaver and Kaufmann. First, we did not intend to imply that our interpretation rested exclusively on the induction of specific repair enzyme systems. Had that been the case we would not have used the phrase "general repair mechanism". Second, with regard to the final statement on the role of O^6 -alkylation of guanine in DNA, we agree that an increased capacity for the repair of O^6 -methylguanine in liver DNA correlates with a high susceptibility to hepatocarcinogenesis in the case of rats chronically exposed to dimethylnitrosamine. Indeed, this may also be the case in our experiments with rats exposed to AAF, although the situation has not been evaluated in our own Wistar rats. While recognising that the role of O^6 -alkylguanine in carcinogenesis is not fully understood it seems unreasonable to consider just two experimental situations (increased O^6 -methylguanine repair after pretreatment and the persistence of O^6 -alkylguanine in the brain of neonatal rats with respect to tumour formation) and not to take into account the considerable information now available (reviewed in refs 2-5). In fact, from reports referred to in these reviews it is clear that careful consideration has been given to the relevance of DNA repair and DNA replication.

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BOOK REVIEWS

Meeting the challenge of eukaryotes

Kenneth Burton

LIKE the well-known first volume that appeared over five years ago (reviewed in *Nature* 252, 145; 1974), this book is a selection of topics written by different authors for the advanced student and aimed at the niche between basic textbooks and specialist reviews. The earlier volume was rather hefty and had mainly general or prokaryotic topics; the new one is of a more reasonable size and its fifteen chapters illustrate the growing confidence of molecular biologists to approach the many challenges posed by eukaryotes.

The authors have generally avoided the temptation to be too comprehensive and sometimes linger to analyse controversies, unanswered problems or the limitations of experimental techniques. I doubt if there can be any biochemist already so knowledgeable over the whole field that he could not find a good number of novel and intriguing topics in the book. Yet no chapter should be beyond the reach of a final-year honours undergraduate, apart from the theory in Blake's article on X-ray crystallography which needs particularly good mathematical preparation. Without it, however, much can still be gleaned including an illuminating discussion of structures derived from single crystal X-ray diffraction. The full appreciation of Tipton's account of the kinetic properties of allosteric and co-operative enzymes also needs a mathematical grasp that is regrettably beyond that of some undergraduate biochemists.

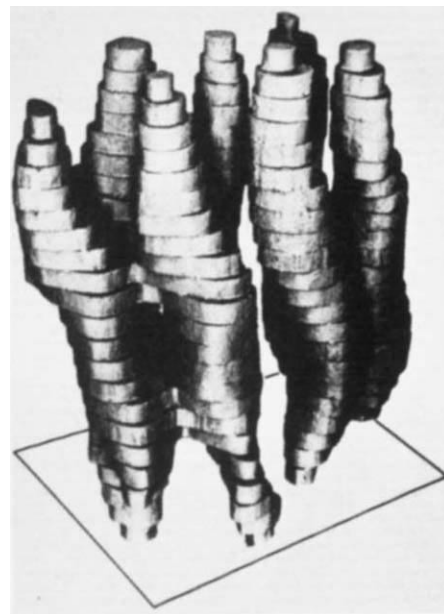
Several other chapters are also of general application to many areas of biochemistry. Nigel Brown knew he had a difficult task to review nucleic acid sequencing, for here the speed of biochemical innovation outpaces the leisurely tread of book publishing. His article was splendid when it was written in

Companion to Biochemistry. Vol. 2. Edited by A.T. Bull, J.R. Lagnado, J.O. Thomas and K.F. Tipton. Pp. 490. (Longman: Harlow, UK, 1979.) £13.95.

1977 but the dideoxynucleotide chain termination method for DNA sequencing and rapid methods for RNA sequencing were not originally included and only get a very brief mention in an addendum. Where space must represent cost, it is surprising to see nearly a page given to the formulae of carbodiimide modification of uracil as well as six pages to the complete sequence of ϕ X174 DNA, however splendid and important the elucidation of that sequence may have been. Glover and Rigby give a lively and stimulating account of gene cloning and recombinant DNA procedures, and how they can be used. As any *Nature* reader will know, this field is also moving forward at a rush aided by the fast methods of DNA sequencing. Again, important applications and developments since the middle of 1977 are not discussed fully though some are mentioned in the inevitable addendum, with only four citations from 1978 and, of course, none from 1979. Thomas clearly expounds the more incisive arguments about the basic molecular structure of chromatin.

Hardy's chapter on ribosomes succeeds in being most useful for the non-specialist but there are other more detailed (and often turgid) reviews on ribosomes elsewhere. Campbell contributes a lucid, well-argued article that is a joy to read on the biosynthesis of proteins destined for secretion. Rumsby's chapter on membranes is less successful: the prose style is guaranteed to switch off all but the most ardent student (for example, "A

consideration of the phase properties of complex lipids is fundamental in understanding current views on the structure of lipids in biological membranes. On heating complex lipids exhibit an intermediate mesomorphic ...'). Moreover, there are unfortunate mistakes in the calculation of protein/lipid ratios in Table 6.3.



Low resolution model of the purple membrane protein, 1975.

Hales, Elder and Davies have chosen to comment on general aspects of clinical biochemistry and the training of clinical biochemists as well as to discuss inherited abnormalities of enzyme function and the use of immunological assays, especially in endocrinology. These discussions are excellent, apart from the lack of up to date references for the last topic.

The remaining chapters are in more specialised fields, such as cell-surface receptors and neurotransmitters (Michell), macromolecular aspects of nervous communication (Lagnado and Beale), complement (Reid), photosynthesis (Gregory) and vision (Voaden). Several of these authors offer aperitifs of relevant background biology that whet the appetite for their main course. Laskey writes most lucidly on the biochemical basis of cell specialisations in early embryonic development. He emphasises DNA

replication, transcription and protein synthesis with an analytical approach that is free from unprofitable speculation. Intermediary metabolism of animals is not given a chapter and plants get only one, on photosynthesis.

I am sure that this book will be read, studied and enjoyed with much profit by advanced students and teachers of biochemistry, even though some chapters are already somewhat obsolete, so raising the question whether quicker ways of production cannot be found for this type of

material. Although there is no use of colour, the presentation is generally good; the type is clear, and there are many good line diagrams and a full subject index. Photographic material is well-chosen, though the quality of reproduction might have been better. The cost per page is not unreasonable by comparison with other publications of similar purpose, such as *Essays in Biochemistry*. □

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Ocean sound speed fluctuations

John A. DeSanto

Sound Transmission through a Fluctuating Ocean. Edited by S. M. Flatte. Pp. 299. (Cambridge University Press: Cambridge, 1979.) £17.50.

THE book is a very well-edited collection of results arising out of the Jason group of the Advanced Research Projects Agency. It presents their theoretical treatment of the effects of a particular model of ocean sound speed fluctuations (due solely to internal waves) on acoustic propagation, and compares the results with those of three experiments, one in the Western Atlantic (Eluthera-Bermuda), one in the Eastern Atlantic (Azores), and one in the North-eastern Pacific (Cobb Seamount).

Both deterministic and statistical properties of the ocean differ from those of other media. Deterministically, the ocean is refractive in depth (unlike atmospheric optics). Statistically, ocean fluctuations differ from those of homogeneous isotropic turbulence, for example, by being anisotropic, inhomogeneous and by having a different spectral function.

The effects on an acoustic signal propagating through the random ocean region depend on the size (diffractive effect) and strength (simply, the rms phase variation) of the random sound speed inhomogeneities. If the region is a weak scatterer ('unsaturated') neither signal amplitude nor phase fluctuate rapidly. For a stronger scatterer ('partially saturated') the phase begins to fluctuate rapidly, and for a very strong scatterer ('saturated') both phase and amplitude fluctuate rapidly. Also, the longer the propagation range and/or the higher the frequency the more the saturation.

The book contains several parts. Part I is on the ocean environment (ocean structure, planetary waves and eddies, and linear internal waves), and is an excellent introduction to the physics of the ocean

and the internal wave fluctuation model used throughout. The fluctuations are thus modelled as arising solely from that spectral window of ocean variability bounded below by the Coriolis (inertial) frequency and above by the Väisälä (buoyancy) frequency.

In Parts II, III and IV, the theoretical methods of sound transmission are developed and interrelated. Path-integral methods (useful in the saturated region), the Rytov approximation (unsaturated region), and the parabolic approximation are all developed, with particular attention paid to ray-theory methods. Of particular use is the discussion of the statistics of acoustic signals in regimes of different saturation.

Do internal waves account for acoustic fluctuations? Part V compares the theoretical predictions to the three experiments. With this extensive and careful development one rather tends to expect a smashing success. It isn't so. The results are mixed. For the (saturated)

Eluthera-Bermuda experiment the rms phase rate and Cartesian signal statistics are predicted well, phase and intensity spectra poorly. For the Cobb Seamount experiment (unsaturated) the internal wave model doesn't account for the measured intensity fluctuations. For the Azores experiment (partial saturation) the agreement is generally good.

My opinion is that the mixed results should not detract from this well written book. It contains a great deal of information, a consistent focused methodology, and the starting point for much additional work. It is suited to an advanced graduate course; and its balance of theory, computations and experimental comparison can serve as a model for extension to the effects of other ocean spectral regimes on acoustic propagation. □

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Geothermal energy

E. R. Oxburgh

Geothermal Energy. By H. Christopher H. Armstead. Pp.357. (E. and F. N. Spon: London, 1978.) £10.50.

OF THE 'alternative energy' resources attracting attention in the early seventies at the time of the rapid rise in world oil prices, geothermal energy is probably the only one for which the expectations are better today than they were five years ago. On the other hand, it must be admitted that at that time expectations were rather low, certainly in Britain.

The exploitation of geothermal energy involves extracting water from sufficiently deep in the ground that it arrives at the surface hot enough for useful heat to be

obtained from it. In geothermal prospecting the aim is to identify places where subsurface temperatures are abnormally high and where, at the same time, the rocks are sufficiently permeable to permit the circulation of water through them. Although in principle any desired temperature up to about 250°C could be reached by deep drilling, in many places this value would be reached only at depths of 10-12 kilometres, close to the depth limits of present drilling technology. Drilling costs are so high, however, that at present the economic viability of a geothermal prospect depends on whether water at useful temperatures can be obtained from depths of less than about 4 kilometres. Of the geothermal power in use today (providing about 0.25% of the world's electrical energy) the greater part is derived from much shallower depths than this; in volcanic areas it is often possible to work with holes of the order of a kilometre deep.

In *Geothermal Energy*, the author touches on every side of the subject. He is clearly an enormous enthusiast and it is his enthusiasm that carries the reader through the book. Indeed in some ways it may be viewed as part handbook and part evangelical tract for the cause of geothermal energy!

The book contains 357 pages of which about 60 are devoted to the general nature of geothermal areas and their geological setting, about 30 to geothermal exploration and drilling, about 160 to the techniques of exploitation and heat extraction at the surface, and about 30 to possible and actual applications of geothermal energy. The remainder is taken up with the index and bibliography (together 25 pages), wide-ranging discussions of problems of pollution, future patterns of world energy usage, and a host of other topics.

There is at present no other single book available with the scope of the present volume, although much of the material is to be found dispersed in an intractable diversity of technical publications. For this reason, if for no other, the book is a valuable one and will be widely used. The author adopts a no-nonsense, down-to-earth, practical approach to the subject which in places is excellent, but

occasionally leads to inaccurate oversimplification. He is at his best when discussing the practical problems and techniques of geothermal field exploitation. Elsewhere he may be idiosyncratic (for example, on page 46, "meteoric waters . . . is merely a 'snob' term for rainwater" — apparently connate water is acceptable); imprecise (for example, on page 85, "... temperature rises less rapidly than depth increases."), or even misleading (on page 44, "... and because of gaping cracks penetrating deeply into the mantle . . ."). This reviewer also feels that there is little point in devoting several pages to fanciful calculations of the total heat content of the Earth, even if the author does at the end emphasise that they are irrelevant to any assessment of exploitable geothermal reserves.

This is a valuable contribution to the literature of the subject. It should be on the shelves of scientific libraries. Its strength and weakness is that it is written by a slightly breathless, uninhibited enthusiast who perhaps oversells his product. □

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How to put your laboratory in order

R.C. Holloway

Laboratory Organization and Management. By F. Grover and P. Wallace. Pp.241. (Butterworth: Sevenoaks, UK.) £5.95.

To many readers of this volume, the thought that they are 'managers' may well be anathema, yet, whether we like it or not, laboratories will no doubt become more organised, like most other institutions. The authors press the point that good management (but not bureaucracy) is a deciding factor between a successful and a poorly organised laboratory. In the present financial climate no doubt this is relevant.

Two chapters, on laboratory planning and service departments, encompass a wide range of subjects in a comparatively short space. There are many practical and useful hints but one is left with the distinct feeling that one has passed over rather too much, too quickly. Certainly, the style tends towards the turgid, with lists sometimes posing as sentences.

Most of the rest is devoted to the fine detail of management. Nearly 30 pages are concerned with the selection and management of staff, from the moment a vacancy

occurs until the time when the appointee leaves the establishment. Choosing the right applicant is undoubtedly a critical matter and mistakes are often made through inexperience, but this chapter reads rather like a miniature course for personnel officers. Store management and laboratory administration are followed by management technique and function. Here we are introduced to forecasting, planning, organising, motivating, controlling and communicating, in considerable detail. As would be anticipated from the authors, the chapter on safety is a model survey of safety organisation and the hazards involved.

Of errors and omissions there are very few. A reference to Dr Hughes' booklet (p.145) on *Design of Radioactive Laboratories* refers to "ionic" instead of "toxic" substances and there is a strange juxtaposition of litres per hour and gallons per day on p.27. A word of warning on present doubts about the safety of biological recirculatory cabinets (p.174) would not have been amiss.

A wealth of material based on the authors' combined experiences is here available for all who wish to embrace the doctrine that management will prevail in the laboratory. So it might, but whether it will lead to better (and happier) laboratories remains to be seen. □

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Biological NMR

Henry Rattle

Biological Applications of Magnetic Resonance. Edited by R.G. Shulman. Pp.595. (Academic: New York and London, 1979.) \$29.

BIOLOGICAL NMR has now reached the stage at which a burgeoning literature provides considerable problems both for those already in the field and for those hoping to enter it. The time is clearly ripe for good review articles, and if these articles are to be of maximum use they must be thoroughly up to date, critically selective in the literature reviewed and accessible to the reasonably knowledgeable but non-specialist reader. All of these requirements are admirably met in the volume under review, but in addition the editor has imposed a pleasingly practical direction on his contributors; for example, the lone article on EPR studies concludes with some brief notes on experimental design for biological materials, and that on ³¹P NMR in living tissue contains useful graphs of the pH-dependence of the chemical shifts of inorganic phosphate as well as design details of the special apparatus used in the experiments. An article on proton nuclear Overhauser effects begins with a very clear and minimally mathematical description of the nuclear Overhauser phenomenon, while one on the use of model compounds in the interpretation of the NMR spectra of haemoproteins starts with a clear table of the oxidation and spin states of iron and a brief qualitative introduction to the theory of hyperfine shifts in paramagnetic systems. Such concern for the non-specialist reader greatly enhances the usefulness of the book.

In all, the volume contains eleven articles averaging some 45–50 pages each. In addition to those already mentioned, they include reviews on hydrogen-bonded proton exchange and its effect on nucleic acid NMR spectra, on nucleic acid structural studies, drug-nucleic acid complexes, anti-body binding sites, multinuclear NMR of the structure of alkaline phosphatase, and the NMR of catalytic groups of serine proteases and of living *Escherichia* cells. All the authors are of high reputation and long experience in biological NMR, and the standard of articles remains high throughout. The volume is reasonably priced by modern standards and is highly recommended to anyone with more than a passing interest in the subject. □

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Long range order in solids

David Sherrington

Long Range Order in Solids. By R.M. White and R.H. Geballe. Pp.409. (Academic: New York and London, 1979.) \$39.50.

THE most obvious example of long range order in solids is the periodic arrangement of atoms which makes up the crystalline state. More generally, however, the concept pervades most of condensed matter physics at low enough temperature. Two other classic manifestations are ferromagnetism and superconductivity, the former relatively easily envisaged as long range order of spin orientation, the latter involving a more subtle order in momentum space. This book is an overview of the whole subject.

There is much common ground among different examples of long range order if one knows how to look. An appreciation of the universality is invaluable in using experience in one area to yield insight in another. One aspect of the book is a discussion of these common features, such as the concepts of order parameters, phase transitions and critical phenomena, the role of symmetry, excitations and so on. The main difference from earlier texts lies, however, in the wealth of experimental data used to illustrate and develop these ideas. Indeed, it is the authors' expressed belief that "a great deal can be learned by looking carefully at experimental data, and that the experience of doing so will develop judgement and intuition". Further believing that "clues to entirely new phenomena are often buried in existing data", they have presented a plethora of data, primarily, but not exclusively, taken from examples in magnetism, superconductivity and charge density wave ordering; within these fields it is difficult to find a topic which has not been touched upon. To complement this data compilation a substance index is included. Unfortunately, however, some potentially useful tables, while indexed under individual materials, are not indexed under the relevant generic names.

The stated level of the book is intermediate between a postgraduate course and a research seminar, and indeed this is about where it lies. Without a reasonably solid base in solid state physics and statistical mechanics the reader would find himself floundering quite often. Elementary many-body theory would be an asset, as also some appreciation of invariants, but one could manage without them. The other essential requirement is determination, because the cover (and often the style) is encyclopaedic but the book seems designed to be read from start to finish rather than

delved into. The student who completes the course will certainly have a broad knowledge at the end. He must, however, be prepared to dig into the research literature to pursue further any individual topic. He will be assisted by the references, which are very up-to-date (several 1978 references).

The book starts by introducing the general concepts of long range order, followed by phenomenological theories of phase transitions and excitations. It then discusses the mechanisms (real and hypothetical) leading to ordering in superconductors and magnets, and the experimental techniques available for probing them. Systematics of long range order in magnetism and superconductivity are followed by discussion of impurity effects and the coexistence of different types of order. In discussing impurity effects there is a divergence from the main subject to that of the formation of local moments in metals and the Kondo effect. Finally, there are chapters on domain structure and inhomogeneous phenomena and on long range order in amorphous and granular materials, these last being very active subjects of current research.

There is sometimes an uneasy mixture of

generality and detail, of different and sometimes inconsistent levels of assumed background knowledge, and of exact and approximate results. I consider it particularly unfortunate that in a book of this type and level approximate results are not always clearly labelled as such. I recommend caution to readers insufficiently experienced to distinguish them.

Overall, the authors have achieved their objective of demonstrating the generality of the concept of long range order, of outlining its language and tools and of showing the importance to its development of the analysis of experiment. This should be useful to a student who has a good basic foundation but lacks breadth. Whether the authors' hope that their organisation of theoretical concepts and experimental manifestations will "enable the reader to make associations which will lead to new ideas" remains to be seen. Let us hope that if it does the new discoverer will let them know. □

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A botanist's what's what

S.M. Walters

Elsevier's Dictionary of Botany 1. Plant Names. By P. Macura. Pp. 580. (Elsevier: Amsterdam and New York, 1979.) \$109.75; Dfl. 225.

THE publication of a new polyglot glossary of botanical words is an event of some importance for librarians and others who face the problems of information retrieval for pure and applied botany. This handsome new volume by Paul Macura of the University of Nevada gives the equivalent in English, French, German, Latin and Russian of more than 6,000 plant names, and a companion volume dealing with terms of a more general nature (excluding Latin) is in preparation.

The Preface claims a unique place in botanical literature for the book, apparently ignoring the existence of the *Botanical Dictionary: Russian-English-German-French-Latin* by N.N. Davidov published in Moscow in 1960. It is true that Macura's dictionary deals exclusively with plant names, for which there are more than three times as many entries as in Davidov; but the proof of the

pudding is in the eating, and in a random test of five English vernacular plant names, birch, bracken, honey fungus, lucerne and maize, Davidov scored four correct or tolerably correct answers, against only three for Macura. Both signally failed to recognise that the English (as opposed to American) word for the crop *Medicago sativa* is 'lucerne', not 'alfalfa'; and Macura seems to think that the English name for *Pteridium aquilinum* is 'adderspit', though he recognises 'bracken' for the western North American var. *pubescens*.

The decision to include in the primary alphabetical listing of English names a large number of "adjectival vernacular names" seems difficult to justify. It enormously expands the book with relatively little gain, and omissions and inclusions seem to be quite arbitrary. Indeed, a more ruthless selection of original material could have produced further economies by eliminating, for example, 'goldmoss' which immediately precedes, and refers directly to, the next entry (No. 2553). The verdict must be that the dictionary is useful, but that it could have been more carefully checked; it could also have been much more compact and therefore cheaper. □

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OBITUARY

R. B. Woodward, 1917–1979

THE sudden death of Robert Burns Woodward of Harvard University on 8 July 1979 at the age of 62, marked the passing of one of the most important organic chemists of this century.

In offering the following account of some aspects of Woodward's achievements the main scientific emphasis will be on his contribution to chemical syntheses. I will leave to others a discussion of the celebrated Woodward-Hoffmann rules. It would, however, be wrong to undertake this exegesis before giving some idea of Woodward's impact on those — and they were hundreds — who came in contact with him.

Woodward's impact on his associates came first from an awesome command of the whole of organic chemistry, ranging from the incredibly complex mass of interrelations and transformations which chemists had produced while unraveling the molecular architecture of complex natural products, such as quinine, morphine or strychnine; to the ever more rapidly accumulating data on the course of chemical reactions and their theoretical basis; extending finally to the multitude of transformations, not excluding the most obscure or esoteric, which might be put to use some day in the rational construction of a complex structure.

Woodward's great strength was his conviction, which he conveyed to his associates, that chemical problems should be placed in a rigorously rational framework; leaving no room for fuzziness, for half understood data, for any but the most mercilessly logical deduction, whether dealing with the structure determination of an unknown natural product, or of an intermediate in a synthetic sequence. Woodward's intense commitment to organic chemistry was allied to the conviction that there must be underlying order beneath what often seemed a chaotic mass of unrelated observations. This approach showed fruit very early (1941) in the well-known "Woodward rules" which provided a generally useful correlation of important structural features of α , β -unsaturated ketones with their ultraviolet absorption maxima.

This early recognition of the importance of the informed application of physical methods to structural problems led Woodward to give a major role to the emerging tool of infrared spectroscopy in his rigorous deduction of the β -lactam structure of penicillin (1944–45). This, and the later conclusion that the vast amount of the existing strychnine chemistry demanded revision of the accepted structure to a different one, now known to be correct, are early examples of



Woodward's mastery of this highly intellectual form of detective work. His skill in presenting his conclusions in a dramatic and lucid style makes his accounts of the elucidation of the structures of strychnine and, *inter alia*, of the tetracyclines or tetrodotoxin fascinating and highly rewarding reading. This is true even today, in spite of the fact that such problems have largely been taken away from chemistry by advances in X-ray crystallography.

The faith in an underlying rational structure showed itself also in Woodward's brilliant use of the "curved arrows" which Robinson's genius had earlier introduced into organic chemistry, to indicate the breaking and forming of bonds, and which have become an essential conceptual tool in visualizing the path from reactants to products. It was Woodward, more than anyone else, not excluding Robinson himself, who showed that Robinson's curved arrows could be submitted to rigorous logical constraints, and that, when they were thus used, they would greatly assist, not only in systematizing observed reactions, but also in predicting which reaction might be possible, and which unlikely. An early example (1948; 1950) of Woodward's command of this tool can be found in his unraveling of some baffling molecular transformations in the Santonin series.

There is one area of chemistry which is most uniquely the domain of organic chemistry, and that is molecular architecture, or synthesis. As chemists entered the twentieth century, the very small number of reactions which might be useful in reaching the complex targets represented by natural products such as cocaine, quinine, morphine or the steroids, coupled with a general lack of knowledge

of ways to control stereochemistry, the three dimensional arrangement of atoms in space, made the challenge of total synthesis of most natural products as formidable as it was exciting. Nevertheless, some beginnings were made: Willstätter synthesized tropinone, the parent of cocaine, around 1900. This was a work of genius, given the state of development of chemical synthesis at that time, but the labored nature of the synthesis also served to emphasize the enormous problems to be faced by would-be molecular architects.

It must have been as important psychologically as it was chemically startling, therefore, when Robert Robinson's famous construction of tropinone burst upon the chemical world in 1917. Willstätter then succeeded in transforming tropinone into cocaine in 1923: the possibility of other complex total syntheses could now be contemplated. Some progress on methodology came from "partial synthesis," the transformation of one natural product into another (e.g. ascorbic acid from hexoses by Haworth, and by Reichstein, in 1933); the forbidding, though achiral, haemin was put together by H. Fischer in 1928 by a combination of steps which must have convinced any would-be practitioner that witchcraft must play a large part in a successful synthesis. Probably the greatest achievements in the decade preceding the second World War were the synthesis of dihydroquinine by Paul Rabe in 1931, and that of equilenin by Bachmann in 1939.

It is on that stage that R. B. Woodward made his dramatic entrance with the announcement of the synthesis of quinine (with W. E. Doering) in 1944. The synthesis commanded worldwide attention because of the burst of publicity (front page of the *New York Times*: "Second Answer to Japan") (the first, being, supposedly, synthetic rubber) which propelled Woodward, then twenty-seven, to a limelight which he did not altogether dislike. Dramatic as it may have seemed to the press, Woodward's quinine synthesis closely followed the scheme laid out by Rabe in his hydroquinine synthesis. It shares with that early synthesis an almost total lack of stereochemical control. And yet, it is of great importance because, in connection with the construction of a piperidine derivative, it introduced what I believe to be one of Woodward's most important contributions to the strategy of complex synthesis: the formation, manipulation and eventual cleavage of rings of carbon atoms as a method of construction of acyclic elements. This approach led to a considerable simplification of the route to the target structure, since it allowed carrying

functional groups in a latent, less reactive form: in the piperidine precursor of quinine, an eventual ϵ -aminoheptanoic acid system is constructed by the cleavage of a 2-methylcyclohexanone, a chemically less reactive, and thus more easily controllable system.

The modern era of concern for stereochemistry started in the period 1950-1955, a period which saw the development of stereospecific, or highly stereoselective, syntheses of morphine, cantharidin, cortisone, cedrol and strychnine. These syntheses were all carried out without taking advantage of the enormous help, just becoming available, of Barton's principles of conformational analysis, but they are important because they represent the first successful efforts to take stereochemistry explicitly into account during the process of synthesis planning. This may seem an obvious prerequisite to a successful synthesis, but one need only read the highly imaginative, though largely inconclusive, series of some sixty papers on steroid "synthesis" by Robinson and his collaborators to see the staggering efforts which could be made to reach synthetic targets (steroids in this case), without any concern for the need to control the relative arrangement of the relevant atoms in space. When one considers that without such control, the connectivity implied by the two-dimensional structure of cholesterol represents 256 different substances, only one of which is cholesterol, it is no wonder that these efforts, brilliant as they sometimes were, remained largely abortive.

Although Woodward's 1951 steroid synthesis was only partially stereoselective in contrast to the cortisone synthesis (by L. H. Sarett) referred to above, it was nevertheless highly influential because it again used the structural simplification made possible by a temporary ring: a cyclohexene ring serves as the source of the steroid D-ring via its cleavage and cyclization to a more functional cyclopentene aldehyde. One also sees here an illustration of another important principle of many Woodward syntheses: the carbon framework is constructed as rapidly as possible, leaving some function (e.g. double bond) which will allow eventual structural adjustments.

It is with the synthesis of strychnine, in 1954, that Woodward began a series of classically elegant syntheses which have not yet been surpassed. In addition to strychnine, they are those of reserpine (1956), of chlorophyll (1960) and of the "western half" of vitamin B₁₂ (1972). In the strychnine construction, the ubiquitous ring element, placed in the α -position of the indole ring of tryptamine, is a dimethoxybenzene ring which is here a latent 1,4 butadienedicarboxylic (muconic) ester system, in a much less reactive form. Before that transformation, however, that same ring is brilliantly used to ensure that an intramolecular cyclization via an

iminium salt occurs at the β -position of an indole ring. The synthetic use of a dimethoxybenzene ring as a precursor of a muconic acid illustrates an aspect of the Woodward mastery which has been referred to previously: the cleavage of an ortho-dimethoxybenzene to a muconic acid had been stored in Woodward's memory since he had noticed its use by Speyer in the degradation of codeine. He was able to recall it just when it would be most dramatically useful.

In the synthesis of strychnine, details of oxidation states were consciously held in the background because of the great structural simplification thus allowed in the construction of the framework. This approach is further refined in the next great synthesis, that of reserpine, in which full and impressive use is made, for the first time, of Barton's conformational principles. In that synthesis, we have, once again, the use of a cyclohexene ring as a surrogate for an eventual aldehyde-ester. We also see here the dramatic simplification produced by the use of a double bond as the precursor of the monomethyl ester of a 1,2-glycol. There were, *a priori*, four possible such systems which might result from the cyclohexene double bond. Woodward had acquired, by that time, extraordinary confidence in his ability to find some way to make molecules dance to his tune: he felt that something might well happen during the synthesis, which would solve this potentially vexing problem. He was not disappointed. Only someone with Woodward's ability to deduce structures from a combination of limited spectral data and mechanistic insight could possibly have capitalised on the remarkable chemical events which provided a solution to the problem. Seldom has it been more true that "chance favors the prepared mind."

Chlorophyll and the "western half" of vitamin B₁₂ will end this broad sketch. In the synthesis of the former, it is still notable that a problem in producing the required regiochemistry was solved by selecting an aminoethyl group as precursor of an eventually required vinyl substituent, and using it in temporary ring formation with a pyrrole aldehyde. Again, the very difficult problem of introducing the two so-called "extra" hydrogens specifically in one of four pyrrole rings was solved, in a unique manner, by a sequence involving cyclization followed by cleavage.

Finally, when we come to the B₁₂ synthesis of 1972, we are treated to a pyrotechnic display of appearing and disappearing rings which are used here to achieve control of almost all the asymmetric centers in the molecule. A propionic acid chain arises from the remains of an anisole ring, and another one from a temporarily constructed cyclohexene. A δ -ketoacid originates from the cleavage of a cyclopentene; an amino acid system is born from a cyclopentanone, as yet further rings make

their tightly orchestrated entrances and exits from the B₁₂ stage: a grand final tribute to the power of this approach to synthesis.

I referred at the very beginning to the great impact that Woodward had on all who associated with him. This was nowhere more obvious than in the celebrated "problem seminars" in which Woodward was always willing to take on all those who cared to match wits and deductive skills with him. It was not just the graduate students and postdoctoral associates who benefitted from contact with Woodward's logical approach to all chemical problems: an impressive number of papers by celebrated chemists all over the world (Arigoni, Bartlett, Barton, Bloch, Djerassi, Eschenmoser, Gates, Inhoffen, Jeger, Klyne, Prelog, Wilkinson, Winstein, Witkop. . .) have Woodward as a coauthor, obvious testimony to the insights that were gained by discussions with him.

Woodward would not have been human had he not enjoyed the attention he commanded. He relished keeping audiences enthralled for hours with a lecture in which he obviously savoured highlighting the already brilliant work he was describing by using meticulously drawn formulas, dramatically enhanced by the controlled use of colored chalk, further to emphasize the image of one in total command of his environment. He was not above playing along with the sensationalism of the media: "That is what the public wants of its heroes"; and he had no doubt that he had earned the right to special recognition at every level. One anecdote, perhaps, sums up the view he had of his rightful place in the world: A new guard at Harvard had just told Woodward that his (blue) car could not be left where he had placed it. "Why is that?" said Woodward. "Your name is not on the list," said the guard. "It isn't?" said Woodward, turning back toward the guard without stopping, "well . . . put it there!" One thing is certain, Woodward's place in chemical history is permanently reserved.

Gilbert Stork

G.S. Forbes

GEORGE SHANNON FORBES, Emeritus Professor of Chemistry at Harvard University, died on 24 June 1979 in his home in Cambridge, Massachusetts.

Born on 17 March 1882 in Boston, Massachusetts, Forbes' interest in science was awakened at the age of eight, by a lecture on pendulums given by his father, George Fairfield Forbes, who was a pioneering teacher of experimental science at the prestigious Roxbury Latin School. But when young Forbes graduated from that same school he aspired to a career as a professor of classics. During his sophomore year at Harvard College, he reports, in a course on qualitative analysis the

challenge of numerous "diabolical mixtures . . . aroused my combative instincts, and provided real excitement . . . Gradually the classics were supplanted by a new enthusiasm. Then followed a truly decisive event. Professor Charles R. Sanger told me that next year's class would be larger than usual. Would I serve as assistant without stipend? Of course I said 'Yes,' and so, not long after my eighteenth birthday, I became a duly appointed officer of instruction in Harvard University, continuing on through senior year . . . Ten years later, this adventure had a direct influence on my fortunes. When Professor Sanger's health became impaired, I was asked to collaborate with him. Upon his death, I took charge of qualitative analysis, and thus my feet became planted upon the academic ladder."

By that time Forbes had long since passed from undergraduate to graduate work at Harvard. Under the direction of T.W. Richards, later to become America's first Nobel laureate in the sciences, Forbes completed in 1905 a doctoral thesis on the electrochemistry of amalgams. He then went on with Richards to a revision of the atomic weights of nitrogen and silver. The keystone of this endeavour was a meticulous redetermination of the exact weight of pure silver nitrate yielded by one gram of pure silver, and the result thus obtained still stands unchallenged (good to 0.002%). But this notable first was also Forbes' last investigation of atomic weights. Most of his nearly 100 research publications deal with solution physical chemistry, especially electrochemistry, and with photochemistry, in which field he was an American pioneer.

Forbes' life and career were wholly centered on Boston and Cambridge Massachusetts. To be sure, he spent 1906-07 at the universities of Leipzig and Berlin, followed by two years as associate in chemistry at Bryn Mawr College. But in 1909 he returned as instructor in physical chemistry to Harvard University, and here he stayed to become Professor in 1926, Chairman of the Chemistry Department in the difficult years 1944-47, and Emeritus Professor in 1948. Even then Forbes' academic career continued for 8 years more Northeastern University and, still persevering as a consultant to chemical industry, inuing as a consultant to chemical industry, he continued laboratory work with his own hands until the age of 90. Triumphant over the hazards besetting the experimental chemist, he was at 97 the oldest living graduate of Roxbury Latin. He outlived his cherished wife of 60 years, *née* Marie Louise Hersey, but is survived by a son, a daughter, and four grandchildren.

Born into a family where father, mother (*née* Elizabeth Shannon), aunts, and uncles were all teachers, Forbes came naturally to that vocation. His teaching career spanned 60 years and some 9000 students. A highly-organized lecturer who wrote everything out in a well-ordered display on the black-

board, Forbes was also a willing spontaneous commentator on the illuminating aberrations of the innumerable lecture experiments he dextrously produced. He was in 1951 the deservedly first recipient of the James Flack Norris Award for outstanding achievement in the teaching of chemistry.

Forbes was a New Englander through and through: sturdy, spare, independent, reserved, and patient. But beneath the somewhat austere exterior lay abundant dry wit, and a human warmth that engaged many friends. An attractively multi-dimensional person, Forbes was a photographer of professional competence, an energetic hiker and climber (long the faculty adviser of the Harvard Mountaineering Club), and an avid musician who lent his (double bass) voice to many local choruses. His high sense of responsibility found expression in a lifetime of devoted service to his science, his Department, his University, and his Church. His was a New England life of little showiness but admirable substance.

Leonard K. Nash

Sir Frank Fraser Darling

THE death of Sir Frank Fraser Darling on 22 October 1979 at the age of 76 will be greatly regretted by naturalists and wildlife conservationists in many parts of the world. Although he only came to wide public notice in Britain when he gave the 1969 Reith Lectures, he had made outstanding contributions to the understanding of wild animals and of the countryside over a period of some 50 years, and his counsels were much sought after in America and elsewhere in the 1950s and '60s, when his own countrymen paid less attention to his opinions.

He started work in 1924 as an agriculturalist. After three years on the staff of the Buckinghamshire County Council he went as a research student to the Institute of Animal Genetics in the University of Edinburgh. He was appointed Chief Officer of the Imperial Bureau of Animal Genetics in Edinburgh in 1930, under the late Professor F.A.E. Crew, and he remained in that post until 1933. He then gave up this urban, academic life, and began his work, often under conditions of considerable austerity, in the highlands and islands of Scotland. He held Carnegie and Rockefeller research fellowships, and was Director of the West Highland Survey from 1944-50, but this was a precarious existence with little of the security scientific workers expect today. However, he was able to spend his time in the field really getting to understand the conditions of his harsh environment.

During this period he wrote many books such as *A Herd of Red Deer* and *A Naturalist on Rona*, vividly describing his

observations and his experience. His *Bird Flocks and the Breeding Cycle* initiated a new field of research. These books had a considerable appeal to other naturalists and to discerning scientists, but were dismissed as too "popular" by the educational establishment, and so did not receive the recognition his admirers felt they deserved. He was bitterly disappointed at the reception given by "the authorities" to his West Highland Survey. His apparently eccentric behaviour and unconventional way of life (something which would be thought normal today) also prevented him from being properly appreciated as an observer and an ecologist in academic circles. At a time when laboratory studies of animal cells or isolated organs were considered more scientific than those involving whole animals in the wild, and when even meticulous observation was considered inferior to experimentation, it is not surprising that he felt he was not properly appreciated.

He did return to academic life for a few years when he was appointed Senior Lecturer in Ecology and Conservation at Edinburgh University in 1953 at the age of nearly 50. He was also invited by the Nature Conservancy in Britain, then a young and developing organisation, to advise them in their studies on Red Deer. This work, and his earlier observations, enabled the Conservancy to introduce a proper management policy particularly to the island of Rhum, where within the National Nature Reserve populations of deer were maintained at optimum levels by a careful culling policy. However, Fraser Darling increasingly found that his views were being taken more seriously in the United States of America, and in 1959 he accepted an invitation from Dr Fairfield Osborn to become Vice President of the Conservation Foundation in Washington DC. His studies of the caribou in Alaska with Dr Starker Leopold, and of the larger mammals in Northern Rhodesia and other African territories did much to consolidate his international reputation.

While in the United States he was impressed with the work done there in setting up National Parks, and he was critical of other countries, including his own, for their slowness in following the American example. However, he praised the British Nature Conservancy for their work, with limited resources, and particularly for establishing several valuable reserves in Scotland.

As already indicated, it was not until 1969 that Britain recognised his contribution to conservation. 1970 had been designated European Conservation Year, and plans to that end were being made by a committee under the patronage of HRH Prince Philip. It was suggested that the 1969 Reith Lectures of the British Broadcasting Corporation should be devoted to some suitable ecological topic, and that they should act as a curtain raiser.

Fraser Darling was an inspired choice as the speaker. He took as his title *Wilderness and Plenty*. The lectures came at the right time, and the lecturer made the most of his opportunity. The public, as distinct from the scientific community, had just learned to use the word "ecology", and to be worried about the future of the environment. The impact of the lectures was considerable, and Fraser Darling was accepted, particularly by students, as a prophet. His status was also radically changed and, somewhat to his surprise, he found himself very much a part of the Establishment. The Queen honoured him with a knighthood, and he was in demand to join the most prestigious Quangos.

Thus from 1970 to 1973 he served on the Nature Conservancy, and in 1970 he was one of the first selected for the Royal Commission on Environmental Pollution. However, he was not seduced by his acceptance by the authorities, and still continued to speak his mind on environmental problems. He was in great demand as a speaker and a writer, but unfortunately ill health prevented him, particularly in recent years, from playing an active part in public life.

It is difficult to assess Fraser Darling's contribution to science at this time. His books show his deep but unsentimental feeling for the countryside and for the animals which inhabit it. He was able to communicate to the public (as in the Reith Lectures), and although this very ability aroused disapproval in some (? envious) academics, it enabled him to make a unique contribution to conservation. However, many of his colleagues, even those who admired him most, did not consider him to be an outstanding ecologist in the academic tradition, and it is not difficult to pick holes in some of his work. Nevertheless he was a leading figure who followed in the British tradition of the study of scientific natural history, and we can take comfort from the fact that at least for the last years of his life this was realised by his countrymen.

Kenneth Mellanby

C.S. Hallpike

DR CHARLES SKINNER HALLPIKE, CBE, FRS, FRCS, FRCP, who died on 26 September 1979, aged 79, was a neuro-otologist of international renown. He was the pioneer in that field and is respected as such in every country of the world.

Although an experienced physiologist and pathologist, Dr Hallpike's special interest was in the relationship of experimental observation to clinical problems. His ability for meticulous work and great clarity of thought enabled him to marshal facts and produce scientific papers in stylish and fluent prose. He is best remembered for his original description of the pathology of Ménière's disease and for the development of a caloric test technique which has, over the years, withstood the

test of time and is now universally accepted.

Dr Hallpike was educated at St Paul's School and graduated in medicine at Guy's Hospital, London in 1926 where he was Entrance Scholar in Arts and Beaney Prizeman in Pathology. His interest in the ear began when he was House Surgeon to the aural departments of Guy's Hospital and Cheltenham General Hospital from 1924 to 1927. Thereafter, as Bernard Baron Research Fellow at the Ferens Institute of Otolaryngology, Middlesex Hospital, the publication of a number of papers concerned with the electrophysiology of hearing resulted in his being awarded the Duveen Travelling Studentship, University of London, 1930 and the Rockefeller Travelling Fellowship 1931, when he studied with Witmark in Germany and in the University of Philadelphia, USA. His high standard of scholarship received further recognition in the awards of the Foulerton Gift Research Fellowship, Royal Society (1937-40) and Gamble Prize in 1934, William J. Muckle Fellowship, University of London, 1941 and Dalby Prize, 1943.

In 1942 he joined the scientific staff of the Medical Research Council at the National Hospital, Queen Square, London where his power of organisation and administration resulted in his appointment as Aural Physician and Director of a newly established Otolaryngological Research Unit at the hospital. Here, with his awareness of the close liaison which should exist between laboratory and clinic in the solution of problems throughout the field of otology, his work became directed towards establishing this link to the great advantage of both. In association with experts in numerous allied disciplines including physics, physiology, biochemistry, histology and clinical investigation, a series of classic publications issued from his unit emphasising the importance of the adoption of quantitative methods in both laboratory and clinical studies of the ear.

The practical outcome of this work included the Medresco hearing aid, the peep-show technique for measuring deafness in young children, recognition of the diagnostic significance of loudness recruitment in sensori-neural deafness and the definition of a number of clinical and pathological entities hitherto grouped together as 'aural vertigo.' An improved technique for temporal bone microtomy was developed, a rotating chair, a head lamp for aural surgery and an ear microscope. Further studies contributed to knowledge of the heredity of labyrinthine disease and the biochemistry of the labyrinthine fluids. At a later stage the narrow-band masking technique for bone conduction audiometry was evolved and an electro-nystagmographic technique, the diagnostic significance of which is still increasing.

Dr Hallpike's unit gained international recognition in the awards he received of the

Gamble Prize for the second time and the Hughlings' Jackson Lectureship and Medal, Royal Society of Medicine 1947; Bárány Medal, University of Uppsala 1958; Guyot Medal, University of Groningen 1959 and the Dalby Prize (awarded jointly) in 1958. In 1956 he became a Fellow of the Royal Society and in 1958 he was honoured with a CBE.

Upon his retirement from the staff of the Medical Research Council, Dr Hallpike was appointed Honorary Aural Physician and Director of Research at the Ferens' Institute of Otolaryngology, Middlesex Hospital from 1965 to 1968. He was a member of numerous learned societies including the Collegium Otorhinolaryngologicum Amicitiae Sacrum whose Shambaugh Prize was awarded to him in 1955. He was an Honorary Fellow of the Royal Academy of Medicine of Ireland, a Fellow of the Royal Society of Medicine, being Honorary Secretary 1938, Editorial Representative 1946 to 1952 and Honorary Member of the Section of Otolaryngology 1970. He was joint Founder of the Bárány Society in 1960, a society which now has world-wide membership and is unique as being solely directed to the study of vestibular problems. Between 1938 and 1965 he was a member of the Flying Personnel Research Committee where his almost unique knowledge of the ear's functioning in health and disease played a large part in overcoming aural problems important in aviation.

Dr Hallpike had the great merit of having inspired many collaborators to engage in scientific work and his wisdom and shrewd judgement will be remembered the world over. He was the acknowledged master of his subject, albeit a hard one, a perfectionist, utterly single-minded in all he undertook. He was quick to recognise and appreciate good work, intolerant of fools but unfailing in giving due credit to his co-workers. The fact that the majority of his unit staff remained with him over the years, a number from its inception until his retirement, is testimony in itself to his integrity. He had enormous courage in the face of an orthopaedic disability resulting from a childhood injury which precluded many outside activities. He played billiards in his younger days and enjoyed rifle practice at Bisley for which he gained a number of awards. He played the piano and violin and was fond of classical music. He enjoyed gardening after his retirement and won prizes locally for his roses. He was a devoted family man and is survived by his wife, Barbara, and two sons.

His death is a great loss to British otology. The establishment of the Medical Research Council Hearing and Balance Unit at the National Hospital, Queen Square, under the directorship of Dr J.D. Hood, with other members of his old staff, remains a living tribute to his pioneer work.

M.R. Dix